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*FLUOROGENIC SUBSTRATES FOR β-D-GALACTOSIDASES AND PHOSPHATASES DERIVED FROM FLUORESCEIN (3, 6-DIHYDROXYFLUORAN) AND ITS MONOMETHYL ETHER**

By Boris Rotman, John A. Zderic, and Marvene Edelstein

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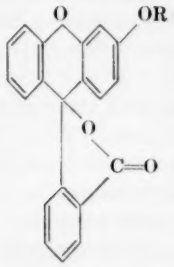
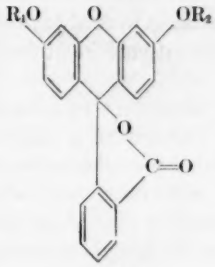
Communicated by Carl Djerassi, May 29, 1963

The sensitivity and usefulness of fluorogenic assays has been demonstrated by measuring the β-D-galactosidase activity of single molecules of this enzyme,[1] individual bacterial cells,[2] and ribosomes.[3]

Syntheses of several new biochemically useful fluorogenic substrates for β-D-galactosidases and phosphatases are described. In addition, evidence is presented that the fluorogenic substrate for β-D-galactosidase, previously believed to be 6-hydroxyfluoran-3-D-galactopyranoside (I*b*),[1, 5] is not a derivative of 6-hydroxyfluoran (I*a*) but rather fluorescein-di-(β-D-galactopyranoside) (II*d*).





I*a* R = H
b R = β-D-galactopyranosyl

II*a* $R_1 = R_2 =$ H
b $R_1 = R_2 =$ Acetyl
c $R_1 = R_2 =$ 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl
d $R_1 = R_2 =$ β-D-galactopyranosyl
e $R_1 = R_2 = PO_3^=$
f $R_1 =$ H, $R_2 =$ Methyl
g $R_1 =$ 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl, $R_2 =$ Methyl
h $R_1 = PO_3^=$, $R_2 =$ Methyl

In 1929, Ghatak and Dutt[4] claimed to have prepared 6-hydroxyfluoran (I*a*), mp 181°, by treatment of 2,4-dihydroxybenzoyl benzoic acid with phenol and concentrated sulfuric acid at 140° for 17 hr. Upon repeating this procedure,[1, 5] it has been possible to isolate an amorphous solid with melting point *ca.* 181°; how-

ever, the lack of reproducibility and low yields of this synthesis prompted a more thorough examination of the reaction. We found that 3,6-dihydroxyfluoran (fluorescein) (II*a*) is formed in this reaction as a major product. At least six other fluorescent compounds were detected.

Fluorescein itself has many polymorphic forms[6, 7] and presents difficulties in terms of positive identification. Its presence in the reaction products was confirmed by direct comparison with authentic fluorescein via paper chromatography, infrared spectroscopy, and by conversion to a pure crystalline diacetate, mp 203–205°. Comparison of this diacetate with authentic fluorescein diacetate[6, 7] (II*b*), by the melting point of a mixture of both compounds, elemental analyses, and spectroscopic analyses, proved the samples to be identical. Moreover, when the sample previously described[1, 5] as 6-hydroxyfluoran-β-D-galactopyranoside (I*b*) was compared by paper chromatography with authentic fluorescein-di-(β-D-galactopyranoside) (II*d*) (see below), both samples were found to have identical mobilities. Likewise, the octa-O-acetyl derivatives of these samples have the same chromatographic behavior, melting point, and infrared spectrum.

Presumably, in the presence of sulfuric acid at 140°, 2,4-dihydroxybenzoyl benzoic acid undergoes decomposition to yield resorcinol which then reacts with the remaining parent molecules to form fluorescein. Direct evidence for this hypothesis rests on the fact that fluorescein production occurs even in the absence of phenol.[7]

The preparation of authentic fluorescein-di-(β-D-galactopyranoside) (II*d*) was carried out by treatment of fluorescein (II*a*) with 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide[8] and silver oxide in benzene solution. The resulting octaacetate (II*c*) was then isolated by column chromatography and hydrolyzed to yield pure II*d*. In addition to the isolation of the octaacetate (II*c*), large quantities of a second substance were always obtained. This highly colored, fluorescent material has been tentatively identified as mono-β-D-galactoside derivative of fluorescein.

It was also of interest to attempt the preparation of the diphosphate (II*e*) of fluorescein to be used as a substrate for phosphatases. When fluorescein was treated with either phosphorous oxychloride in pyridine[16] or cyanoethylphosphate,[9] gross mixtures were obtained, and pure compounds could not be isolated in large amounts. Assuming that these phosphorylations were being complicated by the presence of two hydroxyl functions in fluorescein, we investigated the utility of fluorescein monomethyl ether[10] (3-hydroxy-6-methoxyfluoran) (II*f*), where one of the hydroxyl groups is firmly protected. In this case phosphorylation with phosphorous oxychloride readily yielded the desired derivative (II*h*) which was eventually isolated as the monocyclohexylamine salt. It is of interest to note that, whereas this salt is a colorless solid with good stability at room temperature, the corresponding sodium salt undergoes rapid decomposition in the dry state.

Experimental.—General methods:[17] Thin layer chromatography was carried out on silica gel according to Stahl[11] using benzene:ethyl acetate = 1:3 as the solvent. Descending paper chromatography on Whatman No. 1 or S & S orange was employed with the following solvent systems: solvent I, 1-butanol:pyridine:water = 315:175:240; solvent II, 1-pentanol:1-propanol:water = 40:11:15; solvent III, 2-propanol:ammonium hydroxide (sp. gr. 0.9):water = 7:1:2; solvent IV, 1-propanol:ammonium hydroxide (sp. gr. 0.9):water = 6:3:1; solvent V, isobutyric

acid:1 *M* ammonium hydroxide:0.1 *M* sodium ethylene diaminetetraacetate = 100:60:1.6; solvent VI, ethanol:0.5 *M* ammonium acetate, pH 3.8 = 5:2; solvent VII, ethanol:1 *M* ammonium acetate, pH 7.5 = 5:2.

The chromatograms were examined under long-wave ultraviolet light for fluorescence and then were irradiated with short-wave ultraviolet for a few minutes. Fluorogenic substances were found to give blue fluorescence after this treatment. Phosphates and carbohydrates were detected with enzymes and by spraying with molybdate[12, 13] or ammoniacal silver nitrate,[14] respectively. Rapid examination of column eluants was accomplished by boiling with *1 M* alcoholic potassium hydroxide and visually observing the appearance of fluorescence.

Purification of fluorescein: Commercial fluorescein (Eastman No. 780) was converted to its diacetate[6] and recrystallized three times from ethyl acetate. Hydrolysis was then effected by heating the sample (7.5 gm) for 30 min in 1 *M* alcoholic potassium hydroxide (110 ml). After evaporation to dryness, the residue was precipitated from dilute aqueous potassium hydroxide with 1 *M* hydrochloric acid. Filtration followed by washing with water, and drying gave material of sufficient purity for use in the next step.

Fluorescein-di-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside) (IIc): Purified fluorescein (5 gm, 15 mmole), 2,3,4,6-tetra-O-α-D-galactopyranosyl bromide[8] (12.4 gm, 30 mmole), freshly prepared silver oxide[15] (3.5 gm, 15 mmole), dried benzene (75 ml), and four drops of freshly distilled quinoline were stirred at room temperature in the dark for 60 hr. After removal of the solids by filtration (2.7 gm fluorescein was recovered), the filtrate was washed with 0.5 *M* aqueous sodium hydroxide (3 × 100 ml), and then with water (5 × 100 ml). The solution was then dried over sodium sulfate and, after evaporation to dryness, the residue was taken up in 200 ml of benzene and chromatographed on 100 gm of silicic acid (Mallinckrodt No. 2847) in a 3 × 26 cm column. After washing the column with 200 ml benzene, elution was commenced using a linear gradient of ethyl acetate. The mixing vessel initially contained 700 ml benzene and the reservoir 700 ml benzene–ethyl acetate (4:1). The (4:1) mixture was used for further elution. After eluting with 1100 ml, a peak containing the desired compound was obtained. The solvent was removed under reduced pressure yielding 2.2 gm (2.2 mmole) of material. Two recrystallizations from methanol provided the analytical sample, mp 153–155°, which was colorless and nonfluorescent. λ_{max} (CH_3OH) 223 mμ (log ε 4.72), 273 mμ (3.74); λ_{max} (KBr) 5.70, 6.18, 6.65, 7.28, 8.0–8.3μ; R_f (silica plate) 0.70.

Analysis calculated for C_{48} $H_{48}O_{23}$: C, 58.1; H, 4.9; O, 37.1; CH_3CO, 34.7. Found: C, 58.0; H, 5.2; O, 37.1; CH_3CO, 33.9.

After elution of the column with pure ethyl acetate, 2.5 gm of a crystalline, orange red, strongly fluorescent substance was obtained. This compound is probably a derivative of fluorescein monogalactoside. On the basis of fluorescein, the over-all yield of the reaction was 65%.

Fluorescein-di-(β-D-galactopyranoside) (IId): A solution of 700 mg (0.7 mmole) of IIc in 35 ml of absolute methanol was mixed at 0° with 2 ml of 0.85 *M* sodium methoxide in the same solvent. No starting material could be detected by thin layer chromatography after 15 min. After 2 hr at 0°, the reaction mixture was diluted with an equal volume of water and was passed through Dowex 50W-X8 in the pyridine form. The solution was evaporated to dryness under reduced pressure,

and the residue, dissolved in dioxane and lyophilized, afforded 460 mg (0.66 mmole) of a nonfluorescent yellowish powder (95 per cent yield). The analytical sample, recrystallized twice from ethanol and once from water, was crystalline, colorless, and nonfluorescent. The compound becomes colored when stored in dry form. It is stable in solutions at neutral pH which are kept frozen, mp 202°-dec., λ_{max} (CH_3OH) 224 mμ (log ϵ 4.82), 273 mμ (3.83); λ_{max} (KBr) 5.75, 6.25, 6.70, 7.05 μ; R_f 0.68 in solvent I, 0.17 in solvent II.

Analysis calculated for $C_{32}H_{32}O_{15}.2H_2O$: C, 55.5; H, 5.2; fluorescein, 48.0. Found: C, 55.4; H, 5.3; fluorescein, 47.8.

Fluorescein was determined spectrophotometrically before and after complete hydrolysis of II*d* with β-D-galactosidase. In this way, it was shown that the analytical sample of II*d* contained fluorescence and color corresponding to 0.13% fluorescein. Partial hydrolysis with the enzyme yielded three compounds separated on paper chromatography with solvent II. The R_f of two of these compounds corresponded to those of fluorescein and fluorescein digalactoside. The third compound was probably the monogalactoside of fluorescein.

Fluorescein-3-O-methyl-6-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside) (IIg): Similar treatment of 1.8 gm (5.2 mmole) fluorescein monomethyl ether (II*f*)[10] with 2 gm (4.9 mmole) of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide[8] and 5.8 gm (25 mmole) of Ag_2O,[15] followed by recrystallization from ethyl acetate-hexane, provided 2.4 gm (0.36 mmole) of the title product. In this case, column chromatography was not necessary. The analytical sample was colorless, nonfluorescent, mp 241-242°; λ_{max} (CH_3OH) 224 mμ (log ϵ 4.83), 273 mμ (3.91); λ_{max} (KBr) 5.75, 6.20, 6.32 m, 6.66, 6.78 m, 7.00, 8.0–8.3 μ, R_f (silica plate) 0.75.

The free galactoside, obtained by deacetylation and purification by paper chromatography, was characterized only by enzymatic degradation. This galactoside was found to be less sensitive than II*d* for the fluorogenic assay of β-D-galactosidase.[1]

Monocyclohexylammonium 3-O-methyl-fluorescein phosphate (IIh): A sample of II*f*[10] (5.2 gm, 15 mmole) was dissolved in 50 ml of pyridine, cooled in an ice bath, and then added all at once to two equivalents (2.75 ml) of phosphorous oxychloride in 50 ml of pyridine. After 30 min at 0°, the mixture was poured onto 100 gm of ice, and the solvent evaporated under vacuum at 30°. The residue was dissolved in 200 ml of 2-butanol or 1-butanol (some insoluble material always remains), and extracted five times with 100 ml portions of saturated aqueous sodium sulfate. The organic phase was dried over sodium sulfate and then evaporated to dryness under vacuum at 30°. The residue was dissolved in a minimum of water (*ca.* 50 ml) and the pH carefully adjusted to 7 with 1 *M* sodium hydroxide. Ethanol (*ca.* 100 ml) was then added until precipitation of the sodium salt began. After standing at 0° overnight, the precipitate was collected by centrifugation and washed with a small amount of cold ethanol. The resulting solid could then be stored in the freezer as an ethanolic suspension and under these conditions the salt appears not to undergo any decomposition. If one attempts to store this salt in a dry form, rapid decomposition ensues which is easily detectable by the resulting coloration.

The precipitate obtained from about 5 ml of suspension was dissolved in a minimum volume of water (*ca.* 5 ml) and added to a suspension of Dowex 50W-X8 in the cyclohexylamine salt form (10 ml) in water (10 ml). This mixture was then

stirred for 10 min whereupon a yellow solid precipitated. Methanol (*ca.* 50 ml) was added until the yellow solid had completely dissolved. After filtration, the Dowex resin was washed with 50 ml of methanol and the combined filtrates were evaporated to a small volume (*ca.* 10 ml). The solid, which had then precipitated, was filtered and dissolved in a minimum volume of warm methanol. Ether was then added until precipitation began. After storing overnight at 0°, the precipitate was collected and recrystallized four or five times to obtain a white amorphous solid, nonfluorescent, mp 218–223° dec. Precipitation takes place with difficulty after the first recrystallization and it may be necessary either to evaporate part of the methanol and add more ether, or to leave the solution in the freezer 4–5 days. While this sample appeared homogeneous on Whatman No. 1 paper in solvent systems III–VI, it was noted that on S & S orange paper in solvent system IV a faint bluish streak could be seen following the phosphate spot. In solvent systems III, IV, and VI, a barely perceptible yellow streak may be observed just ahead of the phosphate spot. The intensities of these streaks indicate that they are present only in trace amounts. For analysis, the samples were dried for 3 days at room temperature over phosphorous pentoxide under 0.1 mm pressure.

Analysis calculated for $C_{27}H_{28}O_8NP$: C, 61.7; H, 5.4; N, 2.7; P, 5.9. Found: C, 61.3; H, 5.9; N, 2.8; P, 5.7; λ_{max} (CH_3OH) 224 mμ (log ϵ 4.81), 273 mμ (3.84); λ_{max} (KBr) 5.70, 6.10, 6.20, 6.45 m, 6.65, 6.78 m, 7.02 μ; R_f values 0.49, 0.71, 0.81, 0.74, 0.83 in solvents III, IV, V, VI, and VII, respectively.

The over-all yields for the cyclohexyamine salt were in excess of 10%.

Summary.—Detailed and reproducible procedures are reported for the synthesis of fluorescein-di-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside), fluorescein-di-(β-D-galactopyranoside), 3-O-methyl-fluorescein-2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside, and monocyclohexylammonium 3-O-methyl-fluorescein phosphate. These compounds are important as fluorogenic substrates for determination of β-D-galactosidases and phosphatases because the sensitivity of the assay extends to single molecules of enzyme. The material employed previously, assumed to be 6-hydroxyfluoran-β-D-galactopyranoside, is shown to be fluorescein-di-(β-D-galactopyranoside).

We wish to thank Dr. Koert Gerzon for suggesting that fluorescein could be the product in the condensation of 2,4-dihydroxybenzoyl benzoic acid and phenol. We are also grateful to Dr. John G. Moffatt for his constant interest in this work and invaluable advice, and to Dr. P. G. Holton (Syntex, S.A., Mexico City) for a supply of fluorescein monomethyl ether (II*f*).

* Contribution No. 4 from the Syntex Institute for Molecular Biology.

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[17] All melting points are uncorrected. Microanalyses were performed by Midwest Microlab, Inc. Infrared spectra were determined with a Perkin-Elmer Model 237 spectrophotometer.

THE IONIC CENTRIFUGE CAN GIVE FUSION NUCLEAR POWER

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Different equations for the behavior of plasmas have been given by different authors. Since the results of experiments on the Ionic Centrifuge seem to accord better with one of these than with another, it seems desirable to compare them, particularly as the Ionic Centrifuge seems to offer the possibility of successful nuclear fusion.

1. *The Equations Offered by Spitzer.*—The equations offered by Spitzer in his book[1] (p. 18) are for the positive ions

$$n_i m_i\left(\frac{\partial \mathbf{v}_i}{\partial t} + \mathbf{v}_i\cdot\nabla\mathbf{v}_i\right) = \frac{n_i Ze}{c}(\mathbf{E} + \mathbf{v}_i \times \mathbf{B}) - \nabla\cdot\psi_i - n_i m_i \nabla\Phi + \mathbf{P}_{ie}, \quad \text{Sp(2–4)}$$

$$\mathbf{v}_i = \frac{1}{n_i \Delta V}\sum \mathbf{w}_i, \quad \text{Sp(2–5)}$$

$$\psi_i = \frac{m_i}{\Delta V}\sum(\mathbf{w}_i - \mathbf{v}_i)(\mathbf{w}_i - \mathbf{v}_i), \quad \text{Sp(2–6)}$$

where Φ is the gravitational potential, $\mathbf{v}_i$ the mean velocity of the positive ions in an element of volume ΔV, $\mathbf{w}_i$ the actual velocity of the individual ion, and ψ_i is the stress tensor or dyadic of the ions. $\mathbf{P}_{ie}$ is the total momentum transferred to the ions per unit volume per unit time by "collisions" with the electrons.

The corresponding equations for the electrons are obtained by replacing the quantities $\mathbf{v}_i, n_i, m_i, \psi_i, \mathbf{P}_{ie}$ with $\mathbf{v}_e, n_e, m_e, \psi_e, \mathbf{P}_{ei}$. Z is replaced by -1.

Of course, by Newton's laws, $\mathbf{P}_{ei} + \mathbf{P}_{ie} = 0$, but it cannot be concluded that $\mathbf{P}_{ei} = 0$ and $\mathbf{P}_{ie} = 0$, no matter what the density of the ions and electrons may be, so long as the electric field and magnetic field are determined by the over-all current, $\mathbf{j} = eZn_i\mathbf{v}_i - en_e\mathbf{v}_e$.

Spitzer then gives as an approximate equation (ref. 1, p. 21)

$$\rho\frac{\partial \mathbf{v}}{\partial t} = \mathbf{j} \times \mathbf{B} - \nabla p - \rho\nabla\phi, \quad \text{Sp(2–11)}$$

where

$$\mathbf{v} = \frac{1}{\rho}(n_i m_i \mathbf{v}_i + n_e m_e \mathbf{v}_e), \quad \text{Sp(2–8)}$$

$$\mathbf{j} = \frac{e}{c}(n_i Z \mathbf{v}_i - n_e \mathbf{v}_e), \qquad \text{Sp(2–9)}$$

$$\rho = n_i m_i + n_e m_e. \qquad \text{Sp(2–10)}$$

I am not able to verify the appearance of $\rho(\partial \mathbf{v}/\partial t)$ instead of $\rho(d\mathbf{v}/dt) = \rho(\partial \mathbf{v}/\partial t) + \rho \mathbf{v} \cdot \nabla \mathbf{v}$ on the left of equation Sp(2–11), which otherwise checks with equation (7.47) of the book of Ferraro and Plumpton.[2]

2. *The Equations Offered by Ferraro and Plumpton.*—The equations by Ferraro and Plumpton (quoting from their book,[2] p. 125) are

$$\text{“}\rho_i \frac{d_i \bar{\mathbf{v}}_i}{dt} + \nabla \cdot \mathbf{p}_i - \rho_i \mathbf{F}_i - \nabla \cdot (\rho_i \overline{\mathbf{V}}_i \overline{\mathbf{V}}_i) = \int m_i \mathbf{v}_i \frac{\partial_c f_i}{\partial t} d\mathbf{v}_i. \qquad (7.49)$$

“It is therefore appropriate to introduce the relative pressure tensor $\mathbf{P}_i$ defined by

$$\mathbf{P}_i = \rho_i \overline{\mathbf{V}}_i \overline{\mathbf{V}}_i - \rho_i \overline{\mathbf{V}_i \mathbf{V}_i}, \qquad (7.50)$$

so that equation (7.49) becomes

$$\rho_i \frac{d_i \bar{\mathbf{v}}_i}{dt} + \nabla \cdot \mathbf{P}_i - \rho_i \mathbf{F}_i = \int m_i \mathbf{v}_i \frac{\partial_c f_i}{\partial t} d\mathbf{v}_i. \qquad (7.51)$$

“This corresponds to equation (2.4) page 18 given by L. Spitzer in his book on ionized gases. It should, however, be noted that Spitzer's definition of the pressure tensor does not coincide with that given in equation (7.50), i.e. with the Chapman-Cowling definition.”

Note that $i = (1, 2)$ is a running variable with $i = 1$ for positive ions and $i = 2$ for electrons and that to change from Ferraro and Plumpton's variables to Spitzer's variables,

$$F \,\&\, P \left\{ \begin{array}{l} \mathbf{v}_1 = \mathbf{w}_i \\ \bar{\mathbf{v}}_1 = \mathbf{v}_i \\ \rho_1 = n_i m_i \\ \mathrm{p}_1 = \psi_i \\ \mathbf{F}_1 = \dfrac{Ze}{m_i}(\mathbf{E} + \mathbf{v}_i \times \mathbf{B}) \\ \displaystyle\int n_1 \mathbf{v}_1 \frac{\partial_c f_1}{\partial t} d\mathbf{v}_1 = \mathbf{P}_{ie} \\ \text{omitted} = \Phi \end{array} \right\} Sp. \qquad F \,\&\, P \left\{ \begin{array}{l} \mathbf{v}_2 = \mathbf{w}_e \\ \bar{\mathbf{v}}_2 = \mathbf{v}_e \\ \rho_2 = n_e m_e \\ \mathrm{p}_2 = \psi_e \\ \mathrm{F}_2 = \dfrac{e}{m_e}(\mathbf{E} + \mathbf{v}_e \times \mathbf{B}) \\ \displaystyle\int n_2 \mathbf{v}_2 \frac{\partial_c f_2}{\partial t} d\mathbf{v}_2 = \mathbf{P}_{ei} \end{array} \right\} Sp.$$

$$\mathbf{P}_1 = \mathbf{p}_i - \rho_1 \overline{\mathbf{V}}_1 \overline{\mathbf{V}}_1 \qquad \mathbf{P}_2 = \mathbf{p}_e - \rho_2 \overline{\mathbf{V}}_2 \overline{\mathbf{V}}_2$$

$\mathbf{V}_1$ and $\mathbf{V}_2$ are vectors defined on page 122 by

$$\mathbf{V}_1 = \mathbf{v}_1 - \mathbf{v}_0 \qquad \mathbf{V}_2 = \mathbf{v}_2 - \mathbf{v}_0 \qquad \text{F\&P(7.28)}$$

where the mean mass velocity $\mathbf{v}_0$ is defined by

$$\rho_0 \mathbf{v}_0 = \rho_1 \overline{\mathbf{V}}_1 + \rho_2 \overline{\mathbf{V}}_2 \qquad \text{F\&P(7.27)}$$

where $n_0 = n_1 + n_2 \qquad \rho_0 = \rho_1 + \rho_2$ F&P(7.26)

The following equation is then universally true:

$$\rho_1 \overline{\mathbf{V}}_1 + \rho_2 \overline{\mathbf{V}}_2 = 0. \qquad \text{F\&P(7.29)}$$

Making a few reasonable assumptions as to the nature of the "collisions" between ions and electrons, Ferraro and Plumpton replace the integral expressions on the right of 7.49, getting the equations on page 138.

$$\rho_1 \frac{d_1 \overline{\mathbf{V}}_1}{dt} + \nabla \mathbf{P}_1 - \rho_1 \mathbf{F}_1 = -\frac{\rho_1 \rho_2}{\rho_0 \tau}(\overline{\mathbf{V}}_1 - \overline{\mathbf{V}}_2) \qquad \text{F\&P(7.112)}$$

$$\rho_2 \frac{d_2 \overline{\mathbf{V}}_2}{dt} + \nabla \mathbf{P}_2 - \rho_2 \mathbf{F}_2 = \frac{\rho_1 \rho_2}{\rho_0 \tau}(\overline{\mathbf{V}}_1 - \overline{\mathbf{V}}_2) \qquad \text{F\&P(7.113)}$$

τ is a finite number and equals zero, when $\bar{V}_1 = 0$ and $\bar{V}_2 = 0$.

3. *The First Discharge of the Ionic Centrifuge.*—By the Ionic Centrifuge[3, 4] is meant a central low-voltage arc source of ionization maintained at low voltage at the center of a rather long circular cylinder through which a longitudinal magnetic field passes. The end-plates which bound the vacuum space longitudinally are maintained at one voltage; the cylinder which bounds the space circumferentially is maintained at another voltage. The anode of the arc is taken as zero voltage.

In the first type of discharge, the end plates were given a high enough negative voltage, and the cylinder was allowed to float. To our very great surprise, the floating cylinder voltage rose with the negative end-plate voltage until it reached the Larmor potential for that radius, $-(e/c^2 8m_1)\mathbf{B}^2 r^2$, and beyond that, it stayed constant.[5] The current of ions, uranium mostly, received by the cylinder was the same as was collected on the end-plates in amount. Making $\overline{\mathbf{V}}_1 = 0$, $\overline{\mathbf{V}}_2 = 0$, and therefore $\tau = 0$, at the floating cylinder, we get the constant voltage of $-(e/c^2 8m_1)\mathbf{B}^2 r^2$, with a constant ion current of one half the whole given by the central arc. This result was found when the arc current was varied from one to one hundred amperes, and the magnetic field was varied over a wide range in more than one hundred tests.

These results are all consistent with the equations of Ferraro and Plumpton, and inconsistent with the approximate equation of Spitzer,[1] page 21.

4. *The Second Discharge of the Ionic Centrifuge.*—If we keep the voltage of the end-plates sufficiently high and that of the cylinder low, then presumably the current taken by the end-plates will be low, limited by space charge, and the whole ion current will be received by the cylinder. If the end-plates are energized with a high enough positive potential and if the circular cylinder at low potential is made up of many slats to permit the escape of neutralized positive ions, then presumably we will realize the conditions for nuclear fusion by using a gas capable of nuclear fusion.

Figures 4, 5, and 6 in reference 3 show the presumed currents.

Summary.—The first book[1] gives the general equations of a completely ionized plasma by equations (2–4), (2-5), and (2-6), page 18, agreeing with the second book[2] in equations (7.49), (7.50), and (7.51), page 125. However, the first book, by making an unjustified (to the writer) assumption (ref. 1, p. 20), arrives at an unjustifiable equation (2-11), page 21. The second book makes a justifiable (to the writer) assumption as to the nature of collisions and obtains equations (7.112) and (7.113), page 138. Comparison with the results of experiments on the ionic centrifuge[3, 4] in its first type of discharge over a wide range indicates that the

second book is right in its conclusions. The Ionic Centrifuge in the second type of discharge would seem to offer the possibility of successful nuclear fusion.

[1] Spitzer, L., Jr., *Physics of Fully Ionized Gases* (New York: Interscience Publishers, Inc., 1956).
[2] Ferraro, V. C. A., and C. Plumpton, *Magneto-Fluid Mechanics* (Oxford University Press, 1961).
[3] Slepian, J., these PROCEEDINGS, **47**, 313–319 (1961).
[4] Slepian, J., these PROCEEDINGS, **48**, 913–914 (1962).
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*SUPPRESSOR GENE ALTERATION OF PROTEIN PRIMARY STRUCTURE**

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Some suppressor genes are known to act by restoring an enzymatic activity that is specifically lacking in a mutant strain. This could be accomplished in many ways, with or without the alteration of the enzyme in question.[1–3] Suppressor mutations have been detected which affect the A protein of the tryptophan synthetase of *Escherichia coli*. Previous studies have shown that alterations in the primary structure of this protein can result from forward mutation,[4, 5] reverse mutation,[6] and recombination[6] within the structural gene (the A gene) for this protein. The present paper indicates that a suppressor mutation in a region of the genome distant from the A gene also leads to a change in the primary structure of the A protein.

Pertinent Characteristics of the Tryptophan Synthetase System.—The *Escherichia coli* tryptophan synthetase consists of two separable protein subunits, designated A and B. Together these proteins catalyze the following three reactions:[7] (1) indole + L-serine → L-tryptophan; (2) indoleglycerol phosphate ⇌ indole + 3-phosphoglyceraldehyde; (3) indoleglycerol phosphate + L-serine → L-tryptophan + 3-phosphoglyceraldehyde.[8] Reaction (3) is believed to be the physiologically essential reaction in tryptophan biosynthesis.[7, 9] Many A mutant strains produce an altered A protein, designated A-CRM, which reacts with antibody to the normal A protein.[9] All of the A-CRM's detected to date can combine with the normal B protein component, and this complex can catalyze the In → Tryp reaction, but not the other two reactions, i.e., reactions (2) and (3).

Materials and Methods.—The A mutants and suppressed A mutants listed in this paper were produced by ultraviolet irradiation of the K-12 strain of *E. coli*.[9, 10] The methods employed for the preparation of transducing lysates of phage P1kc and for transduction with this phage have been described previously.[10] All cultures of suppressed A mutants used for the preparation of extracts were examined for possible changes in the cellular population, such as reversion in the A gene, by appropriate plating and transduction techniques.

Enzymatic assays,[7] and procedures for the heat-treatment and acid-treatment of crude extracts,[11] have been described previously. Procedures used for the isolation of the A protein,[12] as well as the methods for the digestion of the protein with

trypsin and chymotrypsin,[13] the isolation of peptides,[13] and the analyses of peptides[4] are described elsewhere.

Results.—Genetic characterization of suppressor mutations: A certain class of tryptophan-independent strains, designated as suppressed A mutants, were isolated in reversion studies performed with various tryptophan synthetase A mutants.[14, 15] These suppressed A mutants were clearly distinguished from revertants which arose by mutation in the A gene. Transduction experiments[15] indicated that the original mutation in the A gene was still present in the suppressed A mutant, and that the site of the suppressor mutation was not linked to the A gene.[16]

Allele specificity tests performed with the suppressor genes in strains A-11 su, A-3 su, and A-36 su indicated that each suppressor gene was allele-specific. Although 50 nonidentical A mutants were examined in tests with each of the suppressor genes, suppression was not detected, i.e., no tryptophan-independent colonies were observed.

Enzymatic characterization of crude extracts of suppressed mutants: Extracts of suppressed mutants differ from extracts of A mutants in that they exhibit low levels of InGP → Tryp activity (Table 1). The level of InGP → Tryp activity restored by a suppressor gene is characteristic of each suppressed mutant. In addition, the ratio of InGP → Tryp activity to In → Tryp activity is quite characteristic for each suppressed mutant, and shows little fluctuation when cultures are grown under similar conditions.

TABLE 1

THE ENZYMATIC ACTIVITIES OF THE A PROTEINS IN EXTRACTS OF VARIOUS MUTANTS AND SUPPRESSED MUTANTS

Strain	A protein-specific activity*		$\frac{\text{InGP} \rightarrow \text{Tryp}}{\text{In} \rightarrow \text{Tryp}}$ per cent
	In → Tryp	InGP → Tryp	
Wild-type	2.5	1	40
A-36	22	0	...
A-36 su	6	0.25	4.2
A-3	35	0	...
A-3 su	44	0.19	0.44
A-11	31	0	...
A-11 su	55	0.48	0.87

* Units/mg protein.

Physical treatments of crude extracts of suppressed mutants: The low level of InGP → Tryp activity detected in extracts of suppressed mutants could be due to an alteration of the CRM molecules, making them all slightly active in this reaction, or it could be due to the formation of a small amount of a second A protein, which was active in the InGP → Tryp reaction. It would be possible to distinguish between these alternatives, if it could be shown that the physical properties of the A protein that is active in the InGP → Tryp reaction differed from the properties of the CRM protein. The three mutants listed in Table 1 were selected for this study because the A-CRM's in each one could be distinguished from the wild-type A protein by differences in heat- or acid-stability. In comparison to the wild-type A protein, the CRM of A-36 is heat-labile, the CRM of A-3 is heat-stable, while the CRM of A-11 is acid-precipitable.[11]

Figure 1 presents the heat inactivation curves for the two activities found in

the crude extracts of two suppressed mutants. The results obtained demonstrate two important facts about the A proteins in these extracts. First, the bulk of the In → Tryp activity in suppressed mutant extracts is associated with an A protein with the same heat sensitivity as the CRM in the original mutant (unsuppressed) extract.[11] Secondly, the A protein active in the InGP → Tryp reaction is clearly different from this CRM in its heat sensitivity, and furthermore resembles the wild-type A protein. As a control for one of the heat inactivation experiments, crude extracts of the wild-type strain and of strain A-36 were mixed to simulate an extract of A-36 su, and a similar heat inactivation experiment performed. The inactivation rates of the two activities in this mixture were the same as those observed with A-36 su extracts.

Heat treatment of an A-11 su extract did not differentially inactivate the InGP → Tryp activity or the In → Tryp activity in this extract. Since the CRM of strain A-11 is as heat-sensitive as the wild-type A protein,[11] no differences would be expected in the two inactivation rates, if the protein bearing the InGP → Tryp activity had the same heat stability as the wild-type A protein. However, acid treatment of an extract of A-11 su clearly showed (Table 2) that the InGP → Tryp activity was associated with a protein that was more acid-stable than the protein bearing the In → Tryp activity, and was similar in behavior to the A protein of the wild-type strain. Here again, the bulk of the A protein resembled the A-CRM found in strain A-11. Similar acid treatment of crude extracts of strains A-36 su and A-3 su led to a loss of activity in both reactions similar to that observed with extracts of the wild-type strain (Table 2). Since the A-CRM's from strains A-36 and A-3 have the same acid stability as the wild-type A protein,[11] it might not be possible to distinguish these A-CRM's from a second A protein, if that protein had the same stability as the wild-type A protein.

TABLE 2

ACID TREATMENT OF EXTRACTS

Extract	Per cent activity remaining*	
	In → Tryp	InGP → Tryp
Wild-type	58	55
A-11	11	...
A-11 su	17, 11	51, 47
A-36	60	...
A-36 su	59	57
A-3	58	...
A-3 su	61	53

* A protein activity in supernatant solutions following acidification of extracts to pH 4.0.

These findings are consistent with the view that the two types of A protein found in extracts of each suppressed mutant are: an A protein active in the InGP → Tryp reaction (hereafter designated as the su-A protein), which has certain physical properties in common with the wild-type A protein; and an A protein which closely resembles the A-CRM of the parental mutant.

Purification and separation of two A proteins by DEAE column chromatography: These findings, as well as previous observations,[2] suggested the presence of a second type of A protein in extracts of suppressed mutants. In further studies with extracts of strain A-36 su, it was possible to separate two A proteins by column

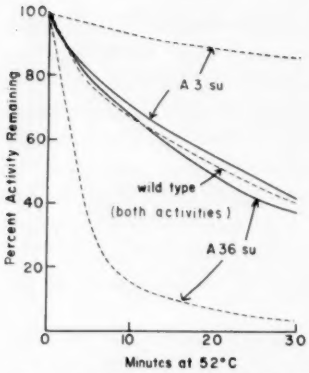

FIG. 1.—Heat inactivation of the A proteins in crude extracts of strains A-36 su, A-3 su, and the wild-type strain. InGP → Tryp activity———, In → Tryp activity - - - - -.

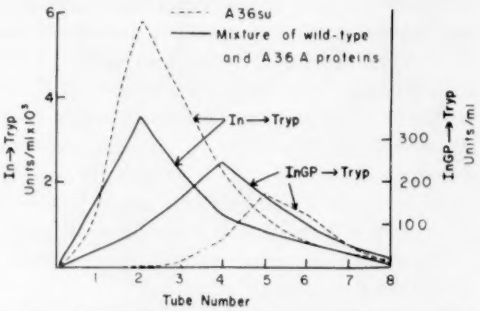

FIG. 2.—Elution pattern from DEAE-cellulose columns of the two A proteins in extracts of strain A-36 su, and of a mixture of the wild-type and A-36 A proteins. A linear phosphate gradient was employed (0.01 *M* → 0.30 *M*, pH 7.0), and fractions of 10 ml were collected. Tube #1 refers to the first tube that contains A protein activity.

chromatography (Fig. 2). As can be seen in this figure, the In → Tryp and InGP → Tryp activity peaks are partially separated.

To determine whether the chromatographic properties of the su-A protein were similar to those of the wild-type A protein, purified A proteins from mutant A-36 and from the wild-type strain were mixed to simulate a purified preparation from strain A-36 su. When this mixture was chromatographed employing the conditions used for the first column procedure (as described above), the In → Tryp and InGP → Tryp activity peaks were partially separated (Fig. 2). As shown also in Figure 2, the activity curves were very similar to those obtained by column chromatography of the preparation from strain A-36 su.

The su-A protein was purified further by combining the fractions containing InGP → Tryp activity from several DEAE columns, concentrating by $(NH_4)_2SO_4$ precipitation, and then rechromatographing on a 100 × 1.2 cm DEAE-cellulose column, using a linear gradient of 0.01 *M* → 0.15 *M* phosphate (pH 7.0). Since some of the early fractions from the previous column with InGP → Tryp activity contained the trailing portion of the In → Tryp activity peak, two activity peaks were also recovered from the second column. However, under the conditions of rechromatography, the two A proteins were completely separated. The purified su-A protein had an InGP → Tryp : In → Tryp activity ratio of 40 per cent, which is identical to that of the wild-type A protein. Heat inactivation studies showed that both of the enzymatic activities of the purified su-A protein were inactivated at the same rate, a rate identical to that for the heat inactivation of the wild-type A protein. These findings indicate that the su-A protein was completely free of the A-CRM protein. Furthermore, a comparison of the specific activities of the A protein peaks with the specific activity of pure wild-type A protein showed that the su-A protein was 50 per cent pure, while the A-CRM was greater than 90 per cent pure.

Analysis of peptides from the A-CRM and su-A proteins of A-36 su: The two A protein fractions isolated from strain A-36 su were examined in peptide pattern

studies. The peptide patterns of a trypsin plus chymotrypsin digest, as well as a chymotrypsin digest of the su-A protein, were found to correspond exactly to the peptide patterns of the wild-type A protein. The peptide pattern of chymotryptic digests of the CRM protein of strain A-36 su showed one difference from a similar wild-type A protein peptide pattern, the position of peptide CP-2, as shown schematically in Figure 3. Although the chymotryptic peptide pattern of the CRM from strain A-36 su differed from the wild-type peptide pattern, it was identical to the peptide pattern of the CRM protein from the original A-36 strain. It had previously been shown that the peptide difference in mutant A-36 was due to the replacement of a particular glycine residue in CP-2 by an arginine residue.[4, 17]

FIG. 3.—A schematic representation of the position of the major peptides in chymotryptic peptide patterns of both the A-CRM and the su-A protein of strain A-36 su. A = the position of peptide CP-2 from the su-A protein, B = the position of CP-2 from the A-CRM. Peptide CP-2 is in position A in wild-type peptide patterns and in position B in peptide patterns of the A-CRM from mutant A-36. The rectangle in the lower left-hand corner indicates the point of application of the sample.

Peptide CP-2 was isolated from the two A proteins from strain A-36 su and its amino acid composition determined. The results of these analyses are compared with the known sequence of amino acids in peptide CP-2 from the wild-type A protein,[17] and with the amino acid composition of CP-2 from A-36 CRM[17] (Table 3). The analyses indicate that peptide CP-2 from the su-A protein has the same composition as the corresponding peptide from the wild-type A protein, while CP-2 from the A-CRM of strain A-36 su has the same composition as CP-2 from the A-CRM of mutant A-36. It is clear from these data that an amino acid replacement occurs in the A protein as a result of the action of the suppressor gene.

TABLE 3

AMINO ACID COMPOSITION OF PEPTIDE CP-2 FROM VARIOUS A PROTEINS

Wild-type	$AspNH_2$-Ala-Ala-Pro-Pro-Leu-$GluNH_2$-*Gly*-Phe
A-36	(Asp*-Ala-Ala-Pro-Pro)-Leu-$GluNH_2$-*Arg*-Phe
A-36 su	
CRM-A	(Asp,* Ala_2, Pro_2, Leu, *Arg*, Glu*)-Phe
su-A	(Asp,* Ala_2, Pro_2, Leu, *Gly*, Glu*)-Phe

* Not determined whether present as amide.

Discussion.—Physical and chemical treatments of extracts of three suppressed A mutants indicate that there are two types of A proteins present in each extract. The majority A protein component is indistinguishable from the original CRM protein, whereas the minority component (the su-A protein) has physical and enzymatic properties characteristic of the wild-type A protein. Peptide pattern studies of the two purified A proteins from strain A-36 su also show that the su-A protein resembles the wild-type A protein, while the CRM protein resembles the A-CRM of mutant A-36. Amino acid analysis of the relevant peptides (CP-2) confirmed the fact that there is a difference in amino acid composition of the two peptides. This fact, in itself, is critical since it indicates that the presence of the

suppressor gene in strain A-36 su has resulted in a change in the primary structure of the A protein.

The amino acid replacement in the A protein due to the suppressor gene of strain A-36 su is an arginine → glycine change in peptide CP-2. Only small amounts of purified su-A protein were available for study, so it was not possible to perform amino acid sequence analyses on peptide CP-2. However, if one makes the plausible assumption that the glycine residue in this peptide occupies the same position as the arginine residue that it replaces, then the effect of this suppressor gene is to restore an amino acid sequence identical to that of the wild-type A protein.[18] The presence of a glycine residue in this peptide is of particular importance in this case, since it has been shown that A proteins with any one of three other amino acids (alanine, serine, and valine) at this position are functional.[6] Although the amino acid found in the wild-type A protein (glycine) was restored in this case, it should be possible for amino acids other than the wild-type amino acid to be inserted at this position. In this regard, it is conceivable that a suppressor mutation could lead to an amino acid replacement in a protein which would not be possible by a single mutational event in the structural gene for this protein.

It has been found that the mutant phenotype of approximately one third of all the A mutants tested can be reversed by suppressor mutations.[15, 19] One mutant which is not suppressible, strain A-46, is particularly interesting in view of the studies reported in this paper. Although extensive reversion analyses and cross-suppression tests have been performed with this mutant, no suppressors have been found which restore InGP → Tryp activity. The amino acid change in the A-46 protein is a glycine → glutamic acid replacement, and this glycine residue is the same one that is replaced by arginine as a result of the A-36 mutation.[5] It is interesting that in the presence of the A-36 suppressor gene, glycine can replace arginine, but not glutamic acid, at this position in the A protein.

The results of these investigations can be most easily explained by postulating that the consequences of a mutation in a suppressor gene such as the suppressor of strain A-36 su is to produce an alteration in the specificity of incorporation of amino acids into proteins. The amino acid changes which result from this type of alteration could be called "mistakes" in protein synthesis. If the cell containing a mutated suppressor gene is incapable of always translating a certain specific DNA nucleotide sequence into the same amino acid without error, then it is important to determine the extent of this error in translation. The term "mistake level" will be used to indicate how often an amino acid at a particular position in a given protein is replaced at the same position by one or another amino acid. This definition does not include as "mistakes" the replacement of an amino acid by its analogues, or the translation of a nonsense sequence of nucleotides into a known amino acid. The "mistake level" might simply be expressed as follows: amino acid X is found to replace Y at a particular position in a protein in 8 per cent of the molecules. The "mistake level" might also be more complex if many amino acids could replace amino acid Y at this particular position and with different frequencies.[20] In any event, a given "mistake level" can be considered to denote a constant probability of misreading the genetic code independent of what proteins, or types of proteins, are being synthesized.

It is not known to what extent the bacterial cell could tolerate many types of

amino acid insertion mistakes. It is probable that very high "mistake levels," single or multiple, if they affected all proteins, would result in lethality. This may explain why all allele-specific suppressors of A mutants examined to date appear to have very low "mistake levels."[21] However, it could be possible to have a fairly high "mistake level" if there were more than one "coding unit" for a particular amino acid (so-called "degenerate coding units"), and if these amino acid coding units were not equally distributed in the DNA. If the suppressor mutation could lead to a translation "mistake" of a minority or infrequently found coding unit, then an amino acid switch found in one protein might not be found in other proteins. In cases where very high levels of an enzyme are restored by the action of a suppressor gene, different interpretations may be applicable.[3]

The finding that some suppressor mutations can reverse the effects of mutations in several genes lends weight to the "mistake in protein synthesis" hypothesis.[22, 23] The fact that a number of different point mutants in the $T4r_{II}$ cistron[22] or in the alkaline phosphatase gene[3] are affected by a single suppressor gene is consistent with this idea and has been interpreted in a similar manner.

There are many ways that the presumed mistake in translation in strain A-36 su might occur. According to our present knowledge of protein synthesis, which

TABLE 4

POSSIBLE SUPPRESSOR "MISTAKES" THAT WOULD LEAD TO THE OCCASIONAL INCORPORATION OF GLYCINE INSTEAD OF ARGININE IN THE A PROTEIN OF STRAIN A-36 su

Mutationally altered component	Effect of alteration	Result
(*a*) Arginine-activating enzyme	Arginine enzyme activates glycine in addition to arginine	Glycine or arginine coupled to same type of arginine-tRNA
(*b*) Glycine-activating enzyme	Glycine enzyme pairs with arginine-tRNA in addition to pairing with glycine-tRNA	Glycine coupled to both arginine-tRNA and glycine-tRNA
(*c*) Arginine-tRNA	Arginine-tRNA pairs with the glycine-activating enzyme in addition to pairing with the arginine-activating enzyme	Glycine or arginine coupled to same type of arginine-tRNA
(*d*) Glycine-tRNA	Glycine-tRNA pairs incorrectly with messenger RNA, in addition to pairing properly	Glycine-tRNA (charged with glycine), pairs with an arginine-coding unit, in addition to pairing with a glycine coding unit in messenger RNA

tRNA = amino acid transfer RNA.

may be incomplete, the possibilities listed in Table 4 can be considered. It is assumed that all of the alterations mentioned lead only to partial losses in specificity. At the present time, the specificities of these components in strain A-36 su are being investigated to determine whether any one of the components has been altered as a result of the suppressor mutation.

We wish to express our thanks to Deanna Thorpe, Patricia Schroeder, and John Horan for their assistance with various aspects of this work.

The following abbreviations were used: Tryp = Tryptophan; In = Indole; InGP = Indoleglycerol phosphate.

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† Predoctoral trainee of the U.S. Public Health Service.

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[16] Preliminary transduction studies indicate that the suppressor gene of strain A-36 su is located close to the B_1 locus on the *E. coli* linkage map.
[17] Carlton, B., and C. Yanofsky, *J. Biol. Chem.*, in press.
[18] It cannot be unequivocally stated that the entire amino acid sequence of the su-A protein is identical to that of the wild-type A protein, since amino acid sequence analyses of every peptide from both of these proteins would be required to establish this point.
[19] Yanofsky, C., D. Helinski, and B. Maling, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 11.
[20] It was thought that perhaps a given suppressor gene, known to cause (for example) a histidine → tyrosine switch in the A protein, could be used as a genetic method for identifying tyrosine → histidine mutational changes in other proteins. However, this may not be possible until it is known whether single or multiple amino acid replacements are involved.
[21] "Mistake levels" in suppressed A mutants are estimated from the ratio of the A protein activity in the InGP → Tryp and In → Tryp reactions. If the A protein active in the InGP → Tryp reaction is similar to the wild-type A protein in its enzymatic properties, then this ratio of enzymatic activities can be taken as an indication of the amount of su-A protein relative to the A-CRM protein.
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*THE EFFECTS OF STREPTONIGRIN ON CULTURED HUMAN LEUKOCYTES**

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Streptonigrin (SN) is a derivative of *Streptomyces flocculus* which has been isolated and screened for its possible antitumor and antibiotic activity. It has not been released for clinical use execpt under experimental conditions. Studies with rodents indicated that it is an extremely cytotoxic compound.[1]

Recent work in this laboratory has shown that SN induces the production of bacteriophage in lysogenic bacteria while inhibiting the net synthesis of bacterial deoxyribonucleic acid (DNA).[2] Furthermore, it causes a marked increase in genetic recombination in mixed bacteriophage infections,[3] and initiates rapid *in vivo* degradation of the DNA of *Escherichia coli*.[4]

Because of these striking effects on macromolecular synthesis, tests of the

activity of SN on human chromosomes seemed warranted. The results to be presented in this communication indicate that, even in extreme dilution, SN induces chromosomal breakage in cultured human leukocytes.

Materials and Methods.—Streptonigrin ($C_{24}H_{20}O_8N_4$), in dry powder form, was obtained from C. Pfizer and Co., Brooklyn, New York. The powder was dissolved in acetone (1 mg/ml) and dilutions were made with sterile distilled water. Various concentrations were added to human leukocyte cultures which had been prepared according to a method similar to that of Moorhead *et al.*[5]

Preliminary experiments, using blood from ten normal individuals, indicated that SN had profound effects on cell growth and chromosome structure. To test the effects of various concentrations for different periods of exposure, the following experiment was performed. Twenty-four treated cultures and two control cultures were established with plasma (30 ml) obtained by a single venipuncture from a 28-year-old normal male. The effects of three concentrations and seven exposure periods were studied according to the regimen outlined in Table 1. Since cells incubated with 0.1 μg/ml for more than 12 hr were destroyed, longer exposure times at this concentration were not tested. Furthermore, since the preliminary work had shown total cell destruction when cultures were treated for the entire 72 hr incubation period with concentrations of SN as low as 0.0001 μg/ml, the maximum period of exposure was limited to 36 hr.

TABLE 1

THREE CONCENTRATIONS OF STREPTONIGRIN INCUBATED WITH LEUKOCYTE CULTURES FOR VARIOUS LENGTHS OF EXPOSURE PRIOR TO HARVEST OF THE CELLS

Concentration of SN (μg/ml)	Hours exposed before harvest of culture						
	2	6	12	18	24	30	36
0.001	−	++	−	++	++	++	++
0.01	++	++	+	++	++	++	−
0.1	+	+	+	−	−	−	−

++ Replicate cultures.
+ Single culture.
− No culture.

Three slides per culture were prepared, coded, and examined by observers who had no knowledge of the treatment. For each slide, the mitotic rate was computed on the basis of the number of division figures observed per 1,000 cells. Ten metaphase cells per slide, selected for adequate chromosome spreading under low power (250×), were analyzed in detail for chromosome number and morphology using oil immersion, phase contrast microscopy (2,500×). Due to the low mitotic rate of the 12 hr exposure to 0.1 μg/ml SN, only 11 analyzable metaphase cells could be found. Each cell thus chosen was scored independently by at least two observers for the presence of chromosomal abnormalities. Only those breaks scored by two observers were counted. Following this initial scoring, 15 additional cells per control slide were examined, bringing the total to 150 control cells analyzed. Chromosomal abnormalities were assigned to seven groups according to the Denver Classification,[6] and where possible, to individual chromosomes. Also, attempts were made to score breaks according to specific regions of the chromosome. A few breaks, rearrangements, and fragments could not be classified.

Results.—Mitotic rate: Statistical analysis of results from replicate cultures exposed to the same treatment showed no significant differences in mitotic rates;

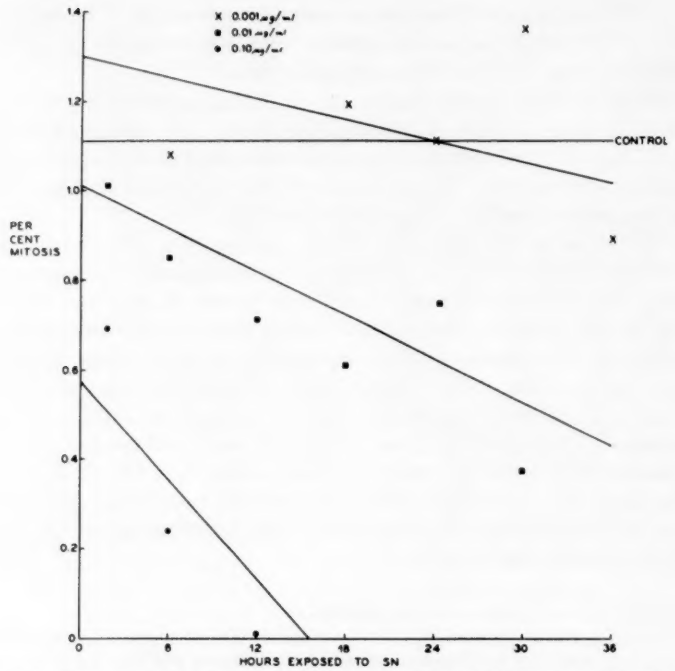

FIG. 1.—The effect on mitotic rate with three concentrations of SN for varying lengths of exposure. The curves drawn are least square lines calculated by regression analysis; each point indicates the average mitotic rate for approximately 6,000 cells.

therefore, these data were pooled. The effect of SN as an inhibitor of mitosis appears related to both concentration and length of exposure (Fig. 1). The control cultures had a mitotic rate of 1.12 per cent. Regression analysis indicated that the mitotic rate of cultures exposed to SN at a concentration of 0.001 μg/ml for 36 hr or less did not differ significantly from that of the controls. However, the mitotic rates of cells exposed to 0.01 and 0.1 μg/ml SN were significantly below the control rates.

Chromosome damage: SN caused much chromosomal breakage and rearrangement. The distribution of chromosome counts in those cells examined in detail appears in Table 2. A modal number of 46 chromosomes was observed in 95.3 per cent of the treated cells. The fact that some cells (3.3 per cent) contained less than 46 chromosomes may be due to random loss of chromosomes caused by rupture of the cell membrane in the preparation of the slide. Counts of greater than 46 chromosomes (1.4 per cent) may be partially attributable to random gain by the

TABLE 2

CHROMOSOME COUNTS IN CELLS EXAMINED FOR CHROMOSOMAL DAMAGE AFTER TREATMENT WITH STREPTONIGRIN

Chromosome number / Number of cells	41	43	44	45	46	47	48	49	Acentric fragments	Total cells
Treated	1	1	4	17	668	6	2	2	46	701
Control	1		2	7	138	2			1	150

breakage of chromosomes with the emergence of fragments or the inclusion of chromosomes from broken cells. Acentric fragments were observed in 46 of the 701 treated cells. The following types of chromosomal abnormalities were produced by SN:

1. Chromatid break—a visual discontinuity of the chromatin material in one chromatid with no apparent strand connecting the broken ends, regardless of the alignment of the two segments (Fig. 2*a*).
2. Isochromatid break—breaks at the same position in both sister chromatids (Fig. 2*b*). Throughout this study isochromatid breaks were scored as a single break.
3. Acentric fragment (Fig. 2*a*).
4. Dicentric chromosome.
5. "Translocation" cross configuration (Fig. 2*c*).
6. Subtelomeric (end-to-end) association of chromosomes (Fig. 2*c*).
7. Cleavage or severe attenuation of the centromere region (Fig. 2*d*).
8. Fusion of the ends of the chromatids of a single chromosome.
9. Increased "stickiness" as evidenced by chromatin strands between chromosomes (Fig. 2*c*).
10. Uncoiling of the chromosomes with long attenuated segments (Fig. 2*d*).
11. Severe fragmentation or degeneration of the entire chromatin material (Figs. 2*e*, 2*f*).

The detailed distribution of breaks by chromosome groups is given in Table 3. A total of 395 breaks and rearrangements of various types was seen in 701 treated cells (0.563 breaks/cell). Four chromatid breaks were observed in the 150 control

TABLE 3

DISTRIBUTION OF CHROMATID AND ISOCHROMATID BREAKS ACCORDING TO THE DENVER CLASSIFICATION OF CHROMOSOME GROUPS

Treatment	Number of breaks by group*								No. of cells examined	Mean number of breaks per cell
	1–3	4–5	6–12, X	13–15	16–18	19–20	21–22, Y	Total		
Control	3				1			4	150	0.027
0.001 μg/ml SN										
6 hr	8	4	6					18	60	0.300
18 hr	5	2	7	1	1			16	60	0.267
24 hr	5	4	9	3	3			24	60	0.400
30 hr	4	4	7	1	2			18	60	0.300
36 hr	4	1	3		1			9	60	0.150
Total:	*26*	*15*	*32*	*5*	*7*			*85*	*300*	*0.283*
0.01 μg/ml SN										
2 hr	4		3					7	60	0.117
6 hr	5	4	5	2	2		1	19	60	0.317
12 hr	7		6	2	1			16	30	0.533
18 hr	10	9	14		4	1	2	40	60	0.667
24 hr	23	13	35	11	2		1	85	60	1.417
30 hr	12	9	29	7	6	1	1	65	60	1.083
Total:	*61*	*35*	*92*	*22*	*15*	*2*	*5*	*232*	*330*	*0.703*
0.1 μg/ml SN										
2 hr	4	2	9		1			16	30	0.533
6 hr	8	3	9		4			24	30	0.800
12 hr	12	4	10	2	3			31	11†	2.818
Total:	*24*	*9*	*28*	*2*	*8*			*71*	*71*	*1.000*
Total	111	59	152	29	30	2	5	388		

* Does not include 7 unclassified breaks.
† Due to low mitotic rate only 11 analyzable cells found.

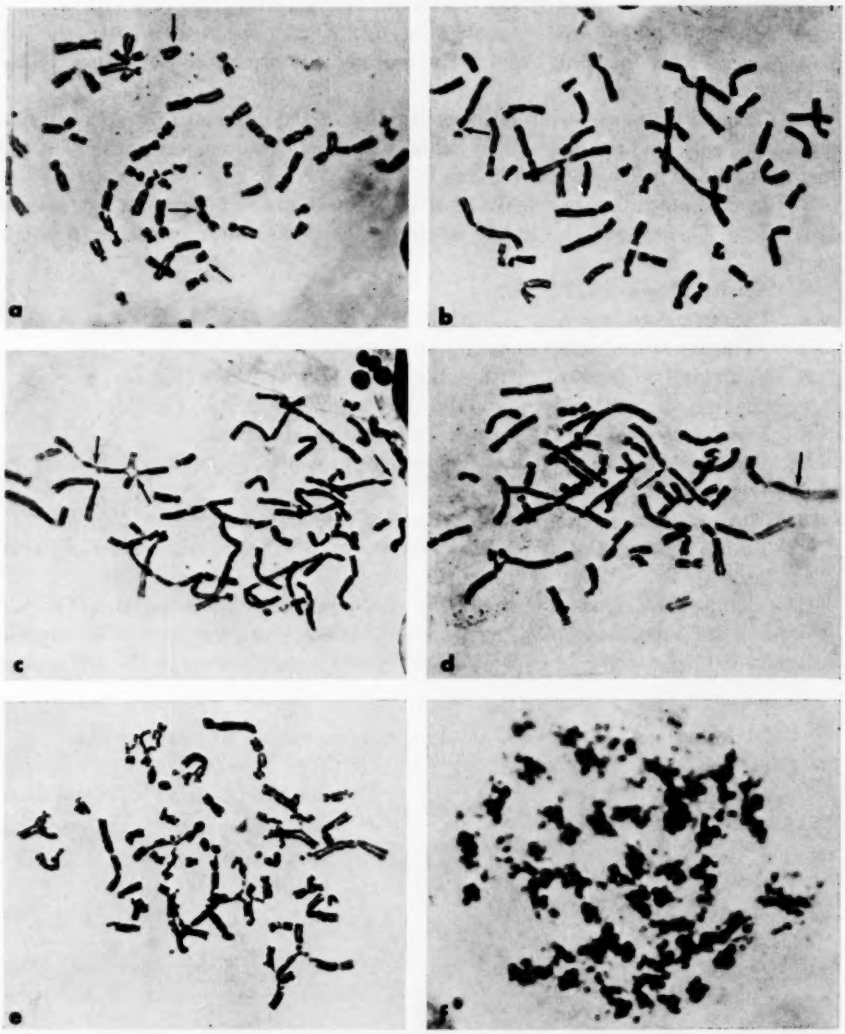

FIG. 2.—Metaphase cells illustrating types of chromosomal damage due to SN. Cells *a* through *e* were treated with 0.01 μg/ml of SN for 24 hr, while cell *f* was treated with 0.1 μg/ml of SN for 6 hr. Arrows indicate type of chromosome damage: (*a*) chromatid break and acentric fragments; (*b*) isochromatid break; (*c*) "translocation" cross, "end-to-end" fusion, and chromatin strand; (*d*) centromere attenuation and uncoiled chromosome segment; (*e*) and (*f*) fragmentation and degeneration of chromosomes.

cells (0.027 breaks/cell). This latter figure compares with frequencies of 0.016 and 0.024 spontaneous breaks per cell in leukocyte cultures reported by Bender and Gooch.[7, 8]

Effects of concentration and length of exposure: Both the mean number of breaks per cell and the percentage of cells with breaks appear to increase with increasing concentrations of SN (Fig. 3). The mean number of breaks per cell was 0.28,

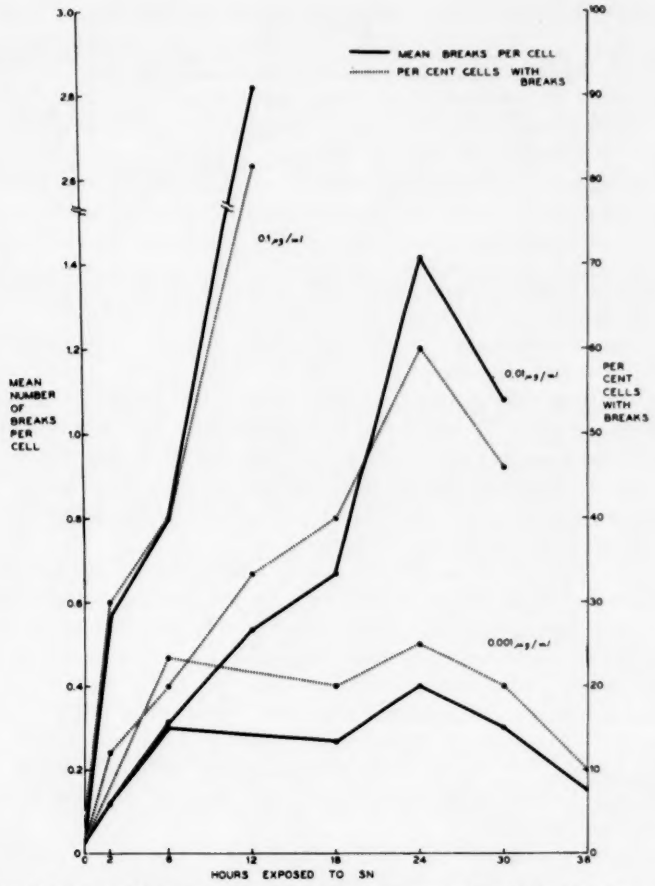

FIG. 3.—The distribution of mean breaks per cell and per cent cells with breaks due to three concentrations of SN for various exposure times.

0.70, and 1.00 for concentrations of 0.001, 0.01, and 0.1 μg/ml of SN, respectively, while 19.7, 35.1, and 42.2 per cent of the cells showed breaks at these same concentrations. An increased number of abnormalities was also observed upon lengthened exposure times. The decrease in breaks following exposures longer than 24 hr is not statistically significant.

Specificity of action: From the distribution in Table 3, SN does not appear to break the chromosomes randomly according to the relative amounts of chromatin material in each of the groups (Table 4: $\chi^2 = 32.19$, $P < 0.005$, $df = 6$). The expected number of breaks in each group was derived from the mean length of each chromosome expressed in units according to the measurements given in the Denver report.[6] The mean lengths of the X and Y chromosomes were added to the doubled mean lengths of the 22 autosomes to obtain the total chromatin material in the normal male somatic chromosome complement. If chromosomal breaks were ran-

TABLE 4

"GOODNESS OF FIT" TEST FOR RANDOM DISTRIBUTION OF CHROMOSOME BREAKS, BASED ON UNIT CHROMOSOME LENGTHS GIVEN IN THE DENVER REPORT[6]

Chromosome group	1–3	4–5	6–12, X	13–15	16–18	19–20	21–22, Y	Total
Observed	111	59	152	29	30	2	5	388
Expected	92.68	47.63	140.54	39.46	33.69	17.79	16.21	388.00
χ^2	3.62	2.71	0.93	2.77	0.40	14.01	7.75	32.19

$P < 0.005$; df = 6.

domly distributed according to length, the expected number of breaks for each group would be obtained by the product of the total number of breaks observed and the relative per cent chromatin in each chromosome group. A deficiency of breaks was noted in the chromosomes of groups 19–20 and 21–22, Y. Only seven breaks were found in these chromosomes, whereas a total of 34 would be expected. No abnormalities were seen in any of the Y chromosomes. Investigation of the randomness of breaks among chromosomes other than the 19–20 and 21–22, Y groups, by assignment of breaks to specific regions of chromosomes which can be individually recognized, is in progress. Additional cells must be scored and precise measurements made before the results are interpreted.

Discussion.—Most of the antibiotics now in widespread clinical use are derived from various species of Streptomyces (e.g., the tetracyclines, erythromycin, mycostatin, and chloramphenicol). It has been shown that certain Streptomyces-derived antibiotics have profound inhibitory influences on the synthesis of nucleic acids and protein. Mitomycin C inhibits bacterial DNA synthesis[9, 10] and its presence in the bacterial cultures leads to a rapid breakdown of DNA to acid-soluble fragments.[11, 12] Actinomycin D depresses mitosis and the metabolism of DNA, RNA, and protein.[13–15] These data, in conjunction with the results presented above, indicate the need for careful studies of the effects of this class of compounds on chromosomal integrity.

Streptonigrin is a recent and exciting addition to the class of antibiotic antimetabolites. The high frequency of chromosomal abnormalities obtained with the minute concentrations used in this study indicates the extreme potency of this agent. Dosages of SN as low as 2.0×10^{-7} *M* caused complete death of the culture in 72 hr, while 2.0×10^{-9} *M* caused 2.82 breaks/cell during a 12 hr exposure. This is in contrast to the effects of 5-bromodeoxyuridine on strain L-M cells in long-term tissue culture, where treatment for 28 days with concentrations of 8.1×10^{-5} *M* produced 1.98 breaks/cell.[16] Changes in chromosomal architecture, like those observed in this study, would most likely lead to reduced viability, if not death of the cell. Inhibition of mitosis and lethal chromosome effects may well be manifestations of the antitumor activity ascribed to SN.

Recently, mammalian chromosome damage of a specific nature has been induced by 5-bromodeoxyuridine[16] and hydroxylamine.[17] The action of SN in causing chromosomal breaks is also nonrandom in the sense that it is not related to unit chromosomal lengths. A relative stability of the chromosomes in groups 19–20 and 21–22, Y, is apparent from the significant deficiency of breaks in these chromosomes. This finding may be partially explicable by increased difficulty in detecting breaks in these smaller chromosomes. Generally, breaks and con-

strictions in chromatids were more frequently visible in those cells with long, uncoiled chromosomes in late prophase or prometaphase (Figs. 2*c*, 2*d*). Upon examining cells with short, compact chromosomes in late metaphase, many breaks seemed either to have "healed" or to have been concealed by the coiling process. This would decrease the probability of identifying breaks in the smaller chromosomes. However, evidence exists which suggests an increased stability of these specific chromosome groups. Karyotypes of aneuploid human cell lines, established from effusions or excised tissues of cancer patients, showed a normal diploid complement for chromosomes of the 21–22, Y group, while the other groups underwent ploidy changes and rearrangements.[18] Karyotypic analyses of a human aneuploid cell line, derived from a biopsy specimen of carcinoma of the cervix, showed disomy of chromosomes 19–20 and 21–22, while all other groups had undergone polysomy and/or rearrangements.[19] This is perhaps surprising in view of the numerous anomalies of chromosome 21 reported in many patients.

The study on genetic recombination in bacteriophage indicates a specific time during the first half of the latent period when SN activity reaches its maximum.[2] Although the present experiment demonstrated an increased effect with increasing exposure times, it would be necessary to design a different experiment to show a specific period of SN activity on leukocytes. Studies utilizing autoradiographic techniques superimposed on SN-treated cultures may be helpful in determining whether the drug acts during the early period of interphase (G_1) or during the period of DNA synthesis (S). Other approaches which may be informative include: (1) "pulsing" of cultures with SN to minimize any possible cumulative effects of long exposures; (2) attempts to synchronize the division of leukocytes. The use of synchronized cultures and/or autoradiographic studies may delineate the specific time of SN activity in the division cycle.

Summary—Streptonigrin is a derivative of *Streptomyces flocculus* with antitumor and antibiotic properties. This drug, when used in extreme dilutions, inhibits mitosis of cultured human leukocytes and causes extensive chromosomal breakage and rearrangements. A variety of chromosomal abnormalities and bizarre metaphase figures were observed. The effect of streptonigrin increased with increasing concentrations as well as lengthening exposure times. Chromosomal damage appears to be nonrandomly distributed with a deficiency of breaks in chromosomes 19, 20, 21, 22, and Y. Approaches to delineate the time of action of streptonigrin in the division cycle are discussed.

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† National Science Foundation Postdoctoral Fellow.

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X-CHROMOSOME MOSAICISM IN FEMALES WITH MUSCULAR DYSTROPHY

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Muscular dystrophy of the childhood or classical Duchenne-type is classified by: (1) the onset of muscular weakness in the pelvic girdle and lower limb muscles, usually before the 7th year of life; (2) appearance almost exclusively in male children; (3) presence of enlargement ("pseudo-hypertrophy") in the calves and deltoids; and (4) a rapid progression of weakness to other muscle groups, leading to death by age 15.

The disease is inherited as a sex-linked trait. Males are affected—females rarely so. The carrier state has been detected in the female in some recent studies which utilized appropriate serum enzyme measurements.[1-4] The dystrophic gene is carried on the X-chromosome and usually only manifests itself in the male. Thus, it is interesting that about 40 females with fairly "classical" Duchenne-type dystrophy have been recorded in the literature.[5] These could be explained by: (*a*) the mating of a carrier female with an affected male; (*b*) the mating of a carrier female with a normal male who had undergone mutation on his X-chromosome; and (*c*) a female with an XO-chromosome complement (Turner's syndrome), the mutant gene being on the single X-chromosome and showing full manifestation. Of these suggestions (*a*) is unlikely, since affected males rarely reproduce; (*b*) is very improbable, since Walton[6] has estimated that this may only occur once in 1,000 million population or one case for every 50,000 dystrophic males; and (*c*) remains a possibility and could only be ruled out by chromatin determinations in these patients. One of the female patients described by Walton[6] may have had an XO-chromosome complement.

Forms of juvenile muscular dystrophy which begin in the lower limbs, as proposed by Becker,[7, 8] include (*a*) malignant X-chromosomal type (Duchenne-type),

(*b*) benign X-chromosomal type, and (*c*) autosomal recessive type which includes the limb-girdle form of others[9-11] and often overlaps the benign X-chromosome type clinically. Discriminant functions based on historical, physical, and laboratory findings have been proposed as a method for improving separation into genetic entities.[12]

Increased serum levels of transaminases, aldolases, and other serum enzymes have been used to confirm the diagnosis of dystrophy or for recognition of preclinical cases.[13] One enzyme, creatine phosphokinase (CPK), is proportionately higher in the serum of dystrophic subjects than are the other enzymes.[3, 4, 14] Recent reports indicate that CPK activity is a fairly sensitive measurement of the carrier state;[4, 15] 15 of 17 females, assumed to be carriers of the gene for Duchenne-type dystrophy, had elevated CPK activity. In families with affected male siblings and Duchenne-type dystrophy, 50 per cent of the unaffected female siblings had elevated CPK activity. The series is small, but fits the expected proportion.[15]

Evidence to be presented suggests that the carrier state in the unaffected female may be identified by CPK elevation and histological changes in the muscle. Further, it is suggested that determination of the carrier state is most reliable in the young child, and that enzyme and histological changes in the muscle in the carrier state signify the existence of a subclinical form of dystrophy. This finding, as well as the infrequent reports of clinically affected females, is best explained on the basis of X-chromosomal mosaicism.

Methods.—CPK measurements were based upon the method described by Tanzer and Gilvarg.[16] Normal values ranged up to 1.5 units. Muscle sections were stained routinely with hematoxylin and eosin and with Azure B at pH 4.0, which specifically colorizes cells blue when RNA is present.[17, 18] Prior treatment with RNAase eliminates all staining in these muscle fibers.

Results.—Enzyme studies: In all, 84 members from 15 families were studied with physical examination, serum CPK values, and muscle biopsies, where appropriate. Each family was identified through an affected male. The values for known carriers, possible carriers, female siblings, and more remote relatives may be grouped as follows:

1. Eight known carrier females: 4 of 8 mothers had elevated serum activity levels for CPK. These mothers were assumed to be carriers if they had (*a*) more than one affected son, (*b*) an affected son and daughter whose CPK was elevated, (*c*) other male members with Duchenne-type dystrophy on the maternal side of the family.

2. Seven possible carrier females: 4 of 7 had elevated CPK values. This includes those mothers with an affected son only, and no daughters with increased CPK activity.

3. Twelve female siblings with dystrophic brothers: 5 of the 12 had elevated CPK levels, a value quite close to the expected proportion of 50 per cent.

4. Six of 25 clinically normal younger brothers with high CPK levels. Muscle biopsy on each revealed the changes of early preclinical muscular dystrophy.[19]

5. Thirty-four clinically normal brothers with normal CPK levels. Muscle biopsies on four of these children were normal.

6. Sixteen aunts, uncles, or cousins of dystrophic males. Three of the males

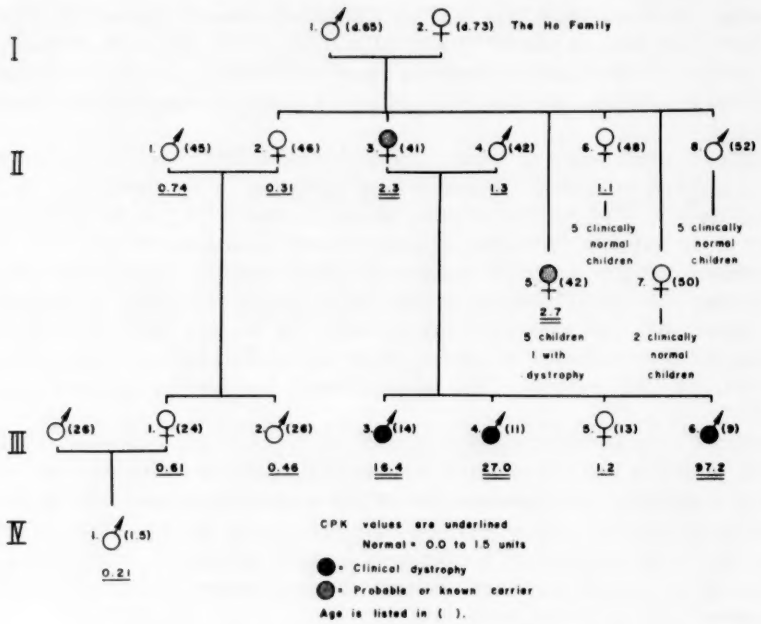
I
II
III
IV
1. (d.65) 2. (d.73) The Ho Family
1. (45) 2. (46) 3. (41) 4. (42) 6. (48) 8. (52)
0.74 0.31 2.3 1.3 1.1
5 clinically normal children
5 clinically normal children
5. (42) 7. (50)
2.7
5 children 1 with dystrophy
2 clinically normal children
(26) 1. (24) 2. (26) 3. (14) 4. (11) 5. (13) 6. (9)
0.61 0.46 16.4 27.0 1.2 97.2
1. (1.5)
0.21
CPK values are underlined
Normal = 0.0 to 1.5 units
= Clinical dystrophy
= Probable or known carrier
Age is listed in ( ).

FIG. 1.

had clinical dystrophy and elevated CPK levels. Three others had "preclinical" dystrophy as proved by muscle biopsy and high CPK levels. Three aunts had moderately elevated CPK and are presumed carriers.

In almost all instances, the CPK levels in females were much lower than in males with clinical dystrophy. Some day-to-day variation was noted, perhaps in part due to previous exercise. Young dystrophic males showed CPK elevations about

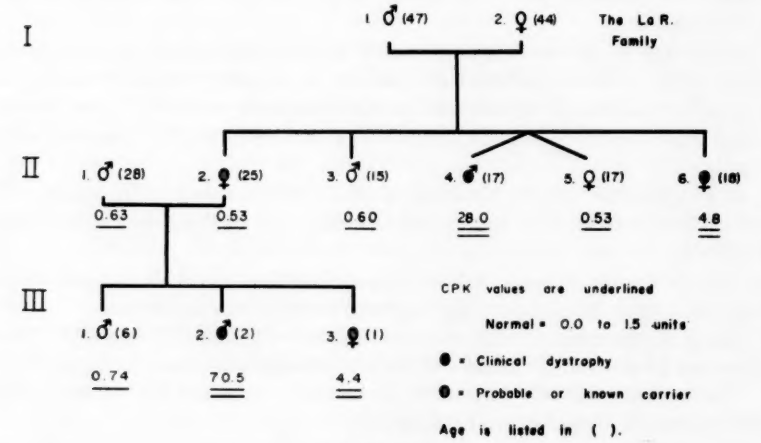
I
II
III
1. (47) 2. (44) The La R. Family
1. (28) 2. (25) 3. (15) 4. (17) 5. (17) 6. (18)
0.63 0.53 0.60 28.0 0.53 4.8
1. (6) 2. (2) 3. (1)
0.74 70.5 4.4
CPK values are underlined
Normal = 0.0 to 1.5 units
= Clinical dystrophy
= Probable or known carrier
Age is listed in ( ).

FIG. 2.

25-fold over normal with a 7–95-fold range (10–140 units). Their clinically normal female siblings, in whom the CPK was elevated, showed increases that averaged 5-fold, range 2- to 60-fold. Among the eleven assumed carrier mothers, the CPK elevations were less, averaging a 2.3-fold increase. The values ranged from 0-, in four mothers with normal values, to a 5-fold increase.

Pedigrees for two of the families are shown in Figures 1 and 2. CPK values are indicated for each member tested. Affected male individuals are readily identified by their high CPK values. The female siblings, such as III 3 in Figure 2, who are presumably carriers, and two heterozygous aunts (II 5, Fig. 1; and II 6, Fig. 2) can be identified by their high values. The CPK value in the mother II 2 (Fig. 2) was normal, whereas II 3 in Figure 1 was elevated. The normal value in the carrier mother will be discussed later in more detail.

FIG. 3.—Essentially normal muscle biopsy from a female sibling of a dystrophic boy. Her CPK value was 97 units. Another biopsy showed focal areas which were typical of the myopathy of dystrophy. H and E, X 164.

Histological studies: Muscle biopsies were obtained from an anterior thigh muscle in three girls, each of whom had an elevation of the CPK level (97 units, 13 units, 8 units). No changes were found in the girl with the lowest CPK value. The patient with the highest CPK value had two biopsies from different areas within the same muscle. One of these showed moderately typical histological features of muscular dystrophy, whereas the other had only subtle alterations with the usual staining procedures (Fig. 3). However, basophilia and, more specifically, the presence of RNA in muscle fibers could be demonstrated by Azure B stains (Figs. 4 and 5). Foci of 3–50 muscle fibers with prominent nuclei were randomly distributed throughout the section, and showed varying degrees of basophilia. Similar histological findings have been noted by us in tissue from males in the preclinical or early stages of muscular dystrophy.[18, 19] The basophilia is interpreted as evidence of a regenerative effort.

In the third girl, with an intermediate elevation of CPK (13 units), changes could not be found after routine staining, but again with Azure B stains, single basophilic fibers or small nests of them, each with prominent nuclei, were found in several areas.

In the first case, the ratio of abnormal to normal fibers was 1:4; in the other case it was 1:90. These were, of course, highly random samplings.

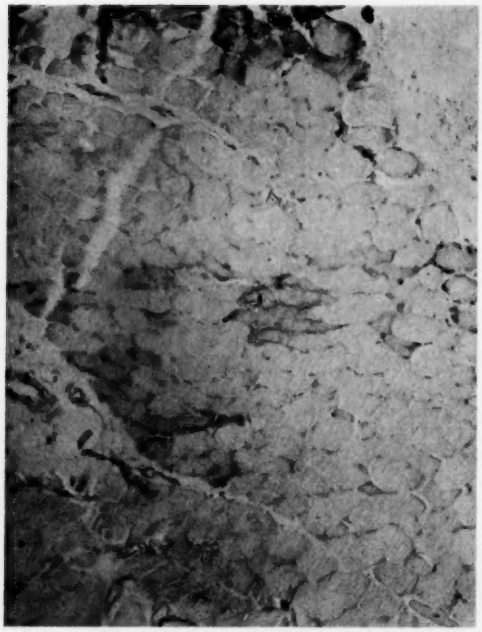

FIG. 4.—Focal faintly and more heavily basophilic clusters of fibers. The section was taken from the same block from which Fig. 3 was sectioned. Azure B, pH 4.0, X 164.

Discussion.—It is apparent that biochemical and histological proof for the carrier state can be determined by the finding of (1) elevated CPK levels, and (2) myopathological changes consisting of basophilic staining of small groups of muscle fibers with prominent nuclei randomly distributed throughout the muscle. Myopathology in the carrier female has not previously been described; it was present in 2 of the 3 patients studied, and suggests that a subclinical form of dystrophy actually exists in the female.

Studies on serum CPK activity reported here, as well as by others, indicate that this determination is of considerable value in detecting the heterozygous female. Approximately 50 per cent of the female siblings in this series and in the series reported by Hughes,[15] had elevated CPK values. This figure closely approximates the expected value of one half for daughters to inherit the mutant gene from their carrier mother.

The expected frequency with which CPK elevations should be found in the carrier mother is 100 per cent. However, only 4 of 8 assumed carrier mothers and 4 of 7 possible carriers had elevated enzyme activity. The mother in Figure 2 (II 2) had a normal value.

This failure to identify the carrier state in II 2, as well as in other mothers, may be explained by the fact that the dystrophic muscle fibers which she may have had during childhood underwent destruction, leaving few, if any, abnormal muscle fibers at this time which could contribute to the serum CPK enzyme pool. This explanation is supported by the finding that in affected males the serum enzyme levels are highest in the preclinical and clinical phases of dystrophy, and tend to fall gradually toward normal values as the muscle wasting advances and the subject becomes confined to bed or a wheelchair.[20]

Previous estimates indicate that about 50 per cent of functional muscle must be lost before any clinical evidence of weakness becomes apparent in carrying out daily activities.[19] Therefore, a carrier female could lose a sizable component of her muscle fibers before weakness became clinically noticeable. In preliminary studies, we have noted that on exercise testing the female carriers will usually perform below the 30th percentile for their sex and age.[21]

It seems reasonable to suggest that the carrier state in Duchenne-type muscular

dystrophy might be more reliably identified by studies on serum CPK activity and muscle tissue in the young female. The use of muscle testing remains to be more fully evaluated.

Recent genetic evidence may also explain the wide variation found in CPK levels in carrier females, as well as suggesting a reason for the number of clinically affected females reported in the literature. This evidence is based on the studies of Ohno and his associates,[22, 23] which revealed that in female somatic cells one X-chromosome becomes heteropyknotic and forms the chromatin or Barr body. Lyon then suggested[24, 25] that: (*a*) the heteropyknotic X-chromosome is genetically inactivated; (*b*) the inactivated X could be either of maternal or paternal origin and is *randomly distributed* throughout different cells; and (*c*) inactivation occurred early in embryonic life, usually by the 12th day in the human embryo.[26, 27] In the male, the X-chromosome never becomes heteropyknotic. The female then is composed of a mosaic of somatic cells, some with a maternal X-chromosome that is functional, and others with a functional paternal X. The female who is heterozygous for a sex-linked gene will show considerable variation in gene expression in her somatic cells depending on the proportion of active normal or active mutant-bearing X-chromosomes that exist.

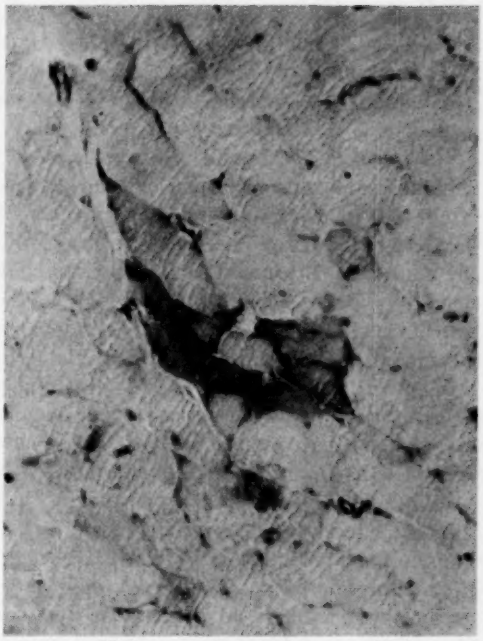

FIG. 5.—Higher magnificant of a group of 7 abnormal fibers as seen in Fig. 4. Note the prominence of the sarcolemmal nuclei and their nucleoli. Azure B, pH 4.0, X 390.

This hypothesis has been supported by studies on coat mottling in the mouse,[25] as well as by certain biochemical studies. Beutler and associates[28] have demonstrated that the red blood cells of females, heterozygous for glucose-6 phosphate dehydrogenase deficiency, consisted of two populations, one normal and the other devoid of G-6 PD enzyme. Recent studies on X-autosome translocations in mice and investigations of G-6 PD activity in the cell clones derived from single cells strongly support the hypothesis that all females are natural mosaics.[29, 30]

This hypothesis can serve to explain the finding of serum enzyme elevations, especially CPK, and the myopathological changes in female carriers of Duchenne-type muscular dystrophy. Hence, a subclinical disorder is present which rarely expresses itself as gross muscular weakness. Two populations of muscle fibers are present, one normal and the other dystrophic. This state is achieved by the operation of X-chromosome mosaicism.

The chance occurrence of lesser degrees of randomization, i.e., a greater number

of normal X-chromosomes, could give rise to totally normal, or nearly so, female carriers. The alternative, i.e., the presence of a greater number of active mutant X-chromosomes, would produce a female with clinical dystrophy. It is suggested that a number of the females reported in the literature with clinical disease may be explained in this manner.

Summary.—(1) Muscular dystrophy often has an onset in the lower limbs in childhood and is usually inherited as a sex-linked recessive trait. It occurs almost exclusively in boys. (2) The abnormal gene is carried in the X-chromosome. A number of cases have been described in girls. A few of these may be explained by unusual genetic combinations, but it would be difficult to explain them all in this manner. (3) Measurements of serum levels of creatine phosphokinase (CPK) can detect all cases of juvenile dystrophy in the clinical or preclinical stages. This enzyme is also moderately elevated in the serum of most carrier mothers and about one half of female siblings, who are presumably also carriers. (4) Since it has been recently shown that all human female somatic cells are distributed in a mosaical pattern, some with an active paternal X-chromosome and others with an active maternal X-chromosome, this hypothesis is evoked to explain both the CPK elevations and some of the cases of dystrophy in females. In the latter, the presumption is made that more maternal (defective) X-chromosomes are represented in the muscle fiber precursor cells than are normal paternal X's. (5) Muscle biopsies from three clinically normal female siblings with elevated CPK levels have shown pathology characteristic of muscular dystrophy in two of them. The pathology was spotty and focal, which was consistent with an admixture of two populations of muscle fibers, both normal and dystrophic. (6) The genetic phenomenon of female X-chromosome mosaicism can explain all of the features noted. Hence, all, or nearly all, carrier heterozygous females *have* muscular dystrophy which is usually subclinical, due to the presence of an adequate complement of normal muscle fibers.

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VARIATIONAL BOUNDARY VALUE PROBLEMS FOR QUASI-LINEAR ELLIPTIC EQUATIONS OF ARBITRARY ORDER

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We consider a quasi-linear elliptic differential operator of order $2m$, $m \geq 1$, in generalized divergence form

$$Au = \sum_{|\alpha|, |\beta| \leq m} D^{\beta}(a_{\alpha\beta}(x, u, \ldots, D^{m}u)D^{\alpha}u) \tag{1}$$

defined on an open subset Ω of Euclidean n-space where the coefficients $a_{\alpha\beta}$ for each choice of the multi-indices α and β are continuous functions of their arguments x, u, and all the derivatives of u up to order m. It is the purpose of the present paper to present in detail a new form of the orthogonal projection method for solving variational boundary value problems for equations of the form (1). This method enables us to obtain weak solutions of such boundary value problems under extremely simple hypotheses on the coefficient functions $a_{\alpha\beta}$, and is an outgrowth and substantial deepening of results obtained by the writer.[1] In that paper, we applied and strengthened a recent theorem due to G. Minty[3] concerning continuous mappings of a Hilbert space into itself of the form $I + F$, where F satisfies a suitable monotonicity condition. The crucial point of our present discussion is the application of results for operators of the form $I + F$, which are no longer continuous but satisfy weaker conditions. This weakening of the continuity requirements makes it possible to apply orthogonal projection techniques directly to equations as general as (1) rather than just to the mildly nonlinear equations discussed in reference 1.

Let us remark that weak solutions of quasi-linear elliptic boundary value problems have been obtained in a series of recent *Doklady* notes by M. I. Vishik (e.g., ref. 6) but under considerably different hypotheses and by a completely different method.

The plan of the present paper is as follows: in Section 1 we define weak solutions for quasi-linear elliptic boundary value problems, state our assumptions, and give the detailed statement of our existence theorems. In Section 2, we give the detailed discussion of the necessary results concerning nonlinear operators in Hilbert space. In Section 3, we apply the abstract results to the proof of our theorems on the existence of weak solutions for boundary value problems.

Section 1.—Let Ω be an open set in the Euclidean space R^n. We write differential operators on R^n in the usual fashion by setting $D^\alpha = \prod_{j=1}^{n} [(1/i)(\partial/\partial x_j)^{\alpha_j}]$ where $\alpha = (\alpha_1, \ldots, \alpha_n)$ is an n-tuple of nonnegative integers, $|\alpha| = \Sigma_j \alpha_j$. The points of Ω will be denoted by $x = (x_1, \ldots, x_n)$, and $\langle u, v \rangle$ will denote the inner product of u, v in $L^2(\Omega)$ with respect to Lebesgue n-measure.

If m is a positive integer, we define $W^{m,2}(\Omega)$ to be the subset of $L^2(\Omega)$ of functions u such that for all α with $|\alpha| \leq m$, $D^\alpha u$ lies in $L^2(\Omega)$. (The derivatives here are taken in the sense of the theory of distributions.) $W^{m,2}(\Omega)$ is a Hilbert space with respect to the inner product and norm

$$(u, v)_m = \sum_{|\alpha| \leq m} \langle D^\alpha u, D^\alpha v \rangle, \qquad \|u\|_m^2 = (u, u)_m.$$

Let $C_c^\infty(\Omega)$ be the family of infinitely differentiable functions with compact support in Ω, $W_0^{m,2}(\Omega)$ the closure of $C_c^\infty(\Omega)$ in $W^{m,2}(\Omega)$.

We consider a quasi-linear elliptic differential operator of order $2m$ of the form

$$Au = \sum_{|\alpha|, |\beta| \leq m} D^\beta(a_{\alpha\beta}(x, u, \ldots, D^m u) D^\alpha u) \tag{1.1}$$

where we assume the following:

ASSUMPTION 1. *The coefficients $a_{\alpha\beta}$ are continuous functions of all their arguments x, u and all the derivatives of u up to order m. They are uniformly bounded for u in $W^{m,2}(\Omega)$ by*

$$|a_{\alpha\beta}(x, u, \ldots, D^m u)| \leq g(\|u\|_m), \text{ a.e. in } \Omega.$$

(The simplest hypothesis ensuring that Assumption 1 holds for any choice of m and n is that $|a_{\alpha\beta}(x, u, \ldots, D^m u)|$ should be bounded functions for all values of their arguments.)

To define a variational boundary value problem for the operator A, we assume that we are also given a closed subspace V of $W^{m,2}(\Omega)$ which contains $C_c^\infty(\Omega)$. For the pair A and V, we make the following basic assumption of strong ellipticity for the boundary value problem:

Let the nonlinear Dirichlet form $a(u, v)$ for the operator A be defined by

$$a(u, v) = \sum_{|\alpha|, |\beta| \leq m} \langle a_{\alpha\beta}(x, u, \ldots, D^m u) D^\alpha u, D^\beta v \rangle$$

for u and v in $W^{m,2}(\Omega)$, a definition which obviously makes sense because of the Assumption 1 on the coefficients $a_{\alpha\beta}$. Then we assume:

ASSUMPTION 2. *There exists a nonincreasing function $c(r)$ of the single real variable r with $\int_1^\infty c(r)\, dr = +\infty$ such that for all u and u_1 in V,*

$$\mathrm{Re}\{a(u, u - u_1) - a(u_1, u - u_1)\} \geq c\,(\max\{\|u\|_m, \|u_1\|_m\}).$$
$$\|u - u_1\|_m^2.$$

The variational boundary value problem for the pair (A, V) is then defined by:

DEFINITION. *Let f be an element of $L^2(\Omega)$. Then u is a weak solution of the equation $Au = f$ satisfying the null variational boundary conditions corresponding to the space V if and only if for all v in V, we have $a(u, v) = \langle f, v\rangle$, while $u \in V$.*

If $V = W_0^{m,2}(\Omega)$, we have the variational Dirichlet problem; if $V = W^{m,2}(\Omega)$, the variational Neumann problem.

The basic result of this paper is the following Theorem whose proof is given in Section 3, using the results of Section 2 on nonlinear equations in Hilbert space:

THEOREM 1. *Let A be a quasi-linear elliptic differential operator of the form (1), V a subspace of $W^{m,2}(\Omega)$ containing $C_c^\infty(\Omega)$. Suppose that A and V satisfy Assumptions 1 and 2 above. Then for every f in $L^2(\Omega)$, there exists one and only one weak solution u of the equation $Au = f$ satisfying the null variational boundary conditions corresponding to the subspace V.*

Section 2.—Let H be a Hilbert space with inner product denoted by (u, v) for u, v in H. We consider a mapping G of an open subset of H into H, where G is neither necessarily linear nor continuous.

DEFINITION. *G is said to be demicontinuous if for any strongly convergent sequence $\{u_k\}$ with $u_k \to u$, $G(u_k)$ converges weakly to $G(u)$ in H.*

LEMMA. *G is demicontinuous if and only if the following condition is satisfied: for any strongly convergent sequence $u_k \to u$ in H, there exists a subsequence of $G(u_k)$ converging weakly to $G(u)$.*

Proof of the Lemma: Obviously, the demicontinuity of G implies the second condition of the Lemma. Suppose, on the other hand, that the second condition holds. Suppose $u_k \to u$ strongly in H. We remark first that $G(u_k)$ is bounded in H, since otherwise we could extract an infinite subsequence with $\|G(u_k)\| \to \infty$ and no subsequence of this subsequence could be weakly convergent. If $G(u_k)$ is bounded in H and if every infinite subsequence has another subsequence converging weakly to $G(u)$, it follows from the weak compactness of closed balls in H that $G(u_k)$ converges weakly to $G(u)$. Thus, the demicontinuity follows, and the Lemma is proved.

THEOREM 2. *Let G be a demicontinuous mapping of the open set D of H into H such that for a positive constant c and all u and u_1 in D, we have*

$$Re\,(G(u) - G(u_1), u - u_1) \geq c\,\|u - u_1\|^2. \tag{2.1}$$

Then, if R is the image of D under G, R is an open subset of H, and G is an open mapping.

COROLLARY TO THEOREM 2. *Suppose that G is a demicontinuous mapping of the open set D into H and that for each point u_0 of D, there exists a neighborhood N and a positive constant c_N such that the inequality (2.1) above holds for all u and u_1 in N. Then the image of D under G is open in H.*

Proof of Theorem 2: Since the restriction to a smaller open set of a mapping G satisfying the inequality (2.1) must also satisfy that inequality, in order to prove Theorem 2 it suffices to prove that the range R of G is open in H.

Let us define the mapping F of D into H by $F(u) = c^{-1}G(u) - u$. Then it

follows from (2.1) that F is a monotone mapping of D into H, i.e., for all u and u_1 in D,

$$Re\ (F(u) - F(u_1),\ u - u_1) \geq 0. \tag{2.2}$$

Moreover, the mapping G and the mapping $c^{-1}G(u) = u + F(u)$ will each have an open range if and only if the other does. We may therefore assume without loss of generality that $G(u) = u + F(u)$ where F is monotone, and that $c = 1$.

It follows from (2.1) that for u and u_1 in D, we have $\|u - u_1\|^2 \leq Re(G(u) - G(u_1),\ u - u_1) \leq \|G(u) - G(u_1)\| \cdot \|u - u_1\|$. It follows immediately that G is one-to-one and that G^{-1} is a contraction, and therefore continuous. We now define the mapping K of the range R of G into H by $K(v) = G^{-1}(v) - F(G^{-1}(v))$. Then:

(a) *K is a contraction:* If v, v_1 lie in R, $y = G(u)$, $v_1 = G(u_1)$, we have: $\|K(v) - K(v_1)\|^2 = \|u - u_1\|^2 + \|F(u) - F(u_1)\|^2 - 2\ Re\ (F(u) - F(u_1),\ u - u_1)$, and $\|v - v_1\|^2 = \|F(u) - F(u_1)\|^2 + \|u - u_1\|^2 + 2\ Re\ (F(u) - F(u_1),\ u - u_1)$, which implies that $\|K(v) - K(v_1)\| \leq \|v - v_1\|$ for all v, v_1 in R.

We now apply a theorem due to Kirszbraun[2] and Valentine[5] which asserts that every (not necessarily linear) contraction defined on a subset of a Hilbert space can be extended to a contraction defined on the whole Hilbert space. (A simple and complete proof of this theorem along lines due essentially to Schoenberg[4] is given in reference 1, Theorem 2.) Let K_1 be a contraction mapping of H into H extending the contraction K, i.e., $K_1(v) = K(v)$ for v in R while for all v in H

$$\|K_1(v) - K_1(v_1)\| \leq \|v - v_1\|.$$

We set $T(v) = {}^1/_2\{v + K_1(v)\}$. Then:

(b) *T is a contraction:* Indeed, we know that

$$Re\ ((v - v_1) - (T(v) - T(v_1)),\ T(v) - T(v_1)) = {}^1/_4\{\|v - v_1\|^2 - \|K_1(v) - K_1(v_1)\|^2\} \geq 0 \tag{2.3}$$

for all v, v_1 in H. Hence, $\|T(v) - T(v_1)\|^2 \leq \|T(v) - T(v_1)\| \cdot \|v - v_1\|$ and the contractiveness of T follows.

(c) *On the subset R, the mapping T coincides with G^{-1}.* For v in R, setting $v = G(u)$, we have

$$v + K_1(v) = v + K(v) = u + F(u) + u - F(u) = 2u,$$

and $T(v) = u$.

(d) *If for a given v in H, $u = T(v)$ lies in D, then $v = G(u)$.* We may choose d so small that any u_1 in H with $\|u - u_1\| < d$ must lie in D. For any $t > 0$ so small that $\|t(v - G(u))\| < d$, the point

$$u_t = u + t(v - G(u))$$

is such a point. We apply the inequality (2.3) of (b) above to the elements v and $v_t = G(u_t)$. We then obtain:

$$Re\ ((v_t - v) - (T(v_t) - T(v)),\ T(v_t) - T(v)) \geq 0,$$

i.e., $Re\ (v_t - v - u_t + u,\ u_t - u) \geq 0$. If we use the fact that $u_t = u + t(v - G(u))$ and that $v_t = u_t + F(u_t)$, we then obtain the inequality:

$$Re\ (F(u_t) + u - v,\ t(v - G(u))) \geq 0.$$

If we remove the positive scalar t, we obtain

$$Re\ (F(u) - v + u,\ F(u) - F(u_t)) \geq \|G(u) - v\|^2.$$

However, the right-hand side of the last inequality is independent of t while on the left, $F(u_t) - F(u) = \{G(u_t) - G(u)\} + \{u - u_t\}$ converges to zero weakly as $t \to 0$ (since $u_t \to u$ strongly in H, and hence, $G(u_t) \to G(u)$ weakly in H by the demicontinuity of G). The left-hand side of the inequality therefore approaches zero, so that $v = G(u)$.

(e) *We may conclude the proof of Theorem 2.* If $u_0 \in D$, $v_0 = G(u_0)$, then $T(v_0) = u_0$. By the continuity of T (which follows from the continuity of the contraction mapping K_1), there exists a neighborhood N_1 of v_0 in H mapped by T into the open set D. For v in this neighborhood, $v = G(T(v))$ by the result of (d) above. Hence, all such v belong to R, R contains N_1, and hence, R is open. QED

THEOREM 3. *Suppose that G is a demicontinuous mapping of the Hilbert space H into itself which satisfies the inequality*

$$Re\ (G(u) - G(u_1),\ u - u_1) \geq c\|u - u_1\|^2 \tag{2.1}$$

for all u and u_1 in H and a fixed positive constant c. Then G is one-to-one and onto H and G has a continuous inverse defined on all of H.

Proof of Theorem 3: By Theorem 2, G is an open mapping of H into H. As we have observed previously, after normalizing to make $c = 1$, G^{-1} is a contraction. Hence, the range of G is closed. Since it is also open and nonempty, the range of G is H, and as already noted, G is one-to-one, and G^{-1} is continuous. QED

THEOREM 4. *Let G be a demicontinuous mapping of the Hilbert space H into itself such that for all u and u_1 in H,*

$$Re\ (G(u) - G(u_1),\ u - u_1) \geq c(\max\{\|u\|, \|u_1\|\}) \cdot \|u - u_1\|^2,$$

where $c(r)$ is a positive nonincreasing function of r for which $\int_1^\infty c(r)\,dr = +\infty$.

Then G is a one-to-one mapping of H onto H and has a continuous inverse defined on all of H.

Proof of Theorem 4: The proof that G is one-to-one and has a continuous inverse defined on its range follows by computation as before. The fact that the range R of G is an open subset of H follows from the Corollary to Theorem 2.

We may suppose without loss of generality that 0 lies in R. It suffices to show that every ray through the origin is contained in R. Suppose v is a point of $H - R$ on a given ray closest to the origin and let $v(t) = tv$, $0 \leq t \leq 1$. For $t < 1$, $v(t) \in R$. Let $u(t) = G^{-1}(v(t))$. The function $u(t)$ cannot converge to a limit in H as $t \to 1$, since for that limit u_1, $G(u_t)$ would converge weakly to $G(u_1)$ which would then have to coincide with v, (contrary to the assumption that v lies in $H - R$). Moreover, $\|u(t)\|$ cannot remain bounded as $t \to 1$, since on every bounded set $\|u - u_1\| \leq c^{-1}\|G(u) - G(u_1)\|$ for a fixed constant $c > 0$, which would imply that the convergence of $v(t) = G(u(t))$ to v would force $u(t)$ to converge as $t \to 1$. Hence, there exists an increasing sequence of parameter values t_k such that $\|u(t_k)\| = k$ and $\|u(t)\| \leq k$ for $t \leq t_k$. Hence, $\|u(t_k) - u(t_{k+1})\| \geq 1$. For this sequence, $\|v(t_{k+1}) - v(t_k)\| \geq c(k+1)\|u(t_{k+1}) - u(t_k)\| \geq c(k+1)$. Therefore, $\Sigma_k c(k) \leq \Sigma_k \|v(t_{k+1}) - v(t_k)\| < \infty$, contradicting the assumption that $\int_1^\infty c(r)dr = +\infty$.

Thus, the assumption that there is a point v in $H - R$ leads to a contradiction, and Theorem 4 is proved. QED

Section 3.—In this final section of the present paper, we shall carry through the proof of Theorem 1 on the existence of weak solutions by translating it into a problem on the solvability of a nonlinear functional equation in the Hilbert space V (considered as a closed subspace of $W^{m,2}(\Omega)$), and solving the latter problem by using the results of Section 2.

Let us remark first that it follows from Assumption 1 that for each pair of elements u and v of $W^{m,2}(\Omega)$,

$$|a(u, v)| \leq g(\|u\|_m)\|u\|_m \cdot \|v\|_m.$$

for fixed u in V, and therefore $a(u, v)$ is a bounded conjugate linear functional of v, which we shall take as running through V. Thus, by the representation theorem for bounded conjugate linear functionals on the Hilbert space V, we have:

DEFINITION. *For each u in V, $G(u)$ is defined to be the unique element of V such that $(G(u), v)_m = a(u, v)$ for all v in V.*

It follows easily that $\|G(u)\| \leq g(\|u\|_m)\|u\|_m$.

LEMMA 1. *It follows from Assumption 2 on (A, V) that for every pair u, v of V, we have*

$$Re(G(u) - G(v), u - v)_m \geq c(\max\{\|u\|_m, \|v\|_m\})\|u - v\|_m^2.$$

Proof of Lemma 1: This is a literal transcription of Assumption 2 after applying the definition of G.

LEMMA 2. *Let f be an element of $L^2(\Omega)$. Then there exists an unique w in V such that $(w, v)_m = \langle f, v\rangle$ for all v in V, and u is a weak solution of the (A, V)-boundary problem for $Au = f$ if and only if $G(u) = w$.*

Proof of Lemma 2: The existence of w and its uniqueness follow from the representation of continuous linear functionals on V. On the other hand, u is a weak solution of the V-problem for $Au = f$ if and only if $u \in V$ and $a(u, v) = \langle f, v\rangle$ for all v in V. Since $\langle f, v\rangle = (w, v)_m$ for all v in V, and $a(u, v) = (G(u), v)_m$, u is a weak solution if and only if $(G(u), v)_m = (w, v)_m$, i.e., $G(u) = w$. QED

LEMMA 3. *Under Assumption 1, the mapping G is a demicontinuous mapping of V into V.*

Proof of Lemma 3: Let $\{u_k\}$ be a sequence with $u_k \to u$ strongly in V. Since $\|u_k\|_m$ are therefore uniformly bounded in k, it follows from Assumption 1 that the functions $a_{\alpha\beta}(x, u_k, \ldots, D^m u_k)$ are uniformly bounded on for all α, β, and k. By the Lemma of Section 2, to prove that G is demicontinuous, it suffices to find a subsequence of $G(u_k)$ which converges weakly. By taking a subsequence of the u_k, we may ensure that for $|\alpha| \leq m$, $D^\alpha u_k$ converges almost everywhere as $k \to \infty$ to $D^\alpha u$. Since the $a_{\alpha\beta}$ are assumed continuous functions of their arguments, it follows that the functions $a_{\alpha\beta}(x, u_k, \ldots, D^m u_k)$ converge boundedly almost everywhere on Ω to $a_{\alpha\beta}(x, u, \ldots, D^m u)$ as $k \to \infty$. Let v be a fixed element of V. We wish to show that $(G(u_k), v)_m \to (G(u), v)_m$. By the definition of G,

$$(G(u_k), v)_m = a(u_k, v), \; (G(u), v)_m = a(u, v),$$

while $$a(u_k, v) = \sum_{|\alpha|, |\beta| \leq m} < a_{\alpha\beta}(x, u_k, \ldots, D^m u_k) D^\alpha u_k, D^\beta v >.$$

For each summand

$$\langle a_{\alpha\beta}(u_k)D^\alpha u_k,\ D^\beta v\rangle = \langle D^\alpha u_k,\ \tilde{a}_{\alpha\beta}(u_k)D^\beta v\rangle,$$

where $D^\alpha u_k$ converges strongly to $D^\alpha u$ in $L^2(\Omega)$. Furthermore, $\tilde{a}_{\alpha\beta}(u_k)D^\beta v$ converges strongly to $\tilde{a}_{\alpha\beta}(u)D^\beta v$ in $L^2(\Omega)$ by dominated convergence. Hence, each term in the sum for $a\ (u_k, v)$ converges to the corresponding term in the sum for $a(u, v)$, $(G(u_k), v)_m \to (G(u), v)_m$ for each v in V, and the proof of Lemma 3 is complete.

Proof of Theorem 1: By Lemma 2, it suffices to show that the mapping G is one-to-one and onto V. By Lemmas 3 and 1, G is demicontinuous and satisfies the inequality (2.4) of Theorem 4 of Section 2. By that Theorem, G is one-to-one, onto, and has a continuous inverse. QED

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PHOTOSYNTHETIC PHOSPHORYLATION CATALYZED BY FACTORS ISOLATED FROM PHOTOSYNTHETIC ORGANISMS,†*

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Communicated by Britton Chance, May 15, 1963

Photosynthetic organisms are of prime importance to life in that they can convert electromagnetic energy into useful chemical energy. It is generally accepted that adenosine triphosphate† is one of the initial stable products of the energy conversion mechanism. Equation 1 summarizes the over-all reaction whereby electromagnetic energy is converted to chemical energy in the form of ATP.

$$\mathrm{ADP} + \mathrm{Pi} \xrightarrow[\substack{\text{Chromatophores}\\ \text{or Chloroplasts}}]{\text{Light}} \mathrm{ATP} \qquad (1)$$

This energy conversion process, which doubtless is multicomponent in nature, is commonly referred to as photosynthetic phosphorylation or photophosphorylation. In view of the importance of this process in cellular metabolism and physiology, it is of considerable interest to determine the naturally occurring components involved in photophosphorylation.

Subsequent to the initial observations of photophosphorylation in bacteria by Frenkel[1] and in higher plants by Arnon *et al.*,[2] a variety of redox substances have been shown to catalyze photophosphorylation.[3] However, many of these substances do not occur in nature. On the other hand, the pyridine nucleotides catalyze a

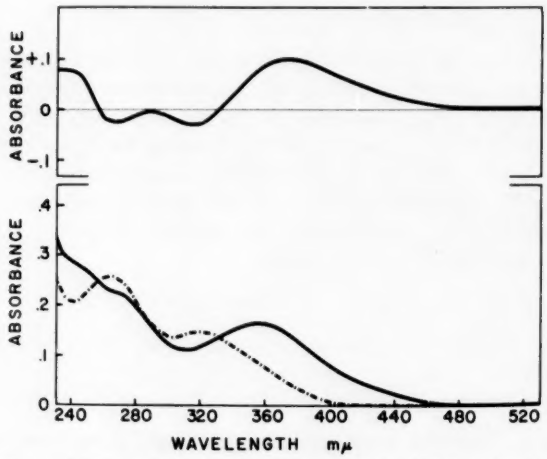

FIG. 1.—Lower portion is absorption spectra of the active factor isolated from spinach chloroplasts. Solid line, factor in 0.1 *N* NaOH; broken line, in 0.1 *N* HCl. Upper portion: difference spectrum.

physiological photophosphorylation in the presence of photosynthetic pyridine nucleotide reductase.[4, 5]

In a search for the components involved in photophosphorylation by spinach chloroplast fragments, we observed that ATP production in the presence of NADP was stimulated much more than was NADP reduction. Further study revealed that this additional ATP production was catalyzed by a water-soluble, heat-stable factor(s) which was quite active in the absence of other exogenous redox substances. Evidence is presented that this factor(s) occurs in all types of photosynthetic organisms. Furthermore, the factor from one type of organism catalyzes photo-

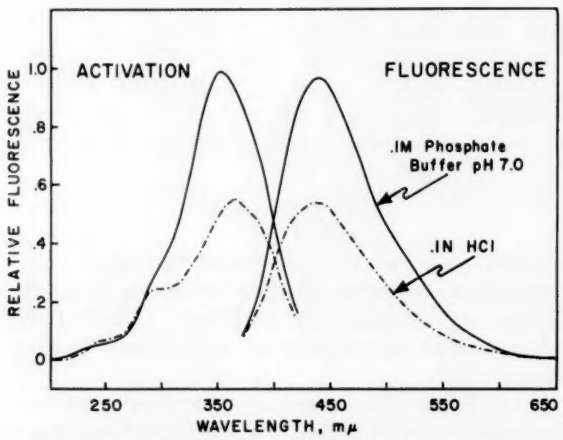

FIG. 2.—Activation and fluorescence spectra of the active factor from spinach chloroplasts, 0.2 mg/ml. Slit arrangement No. 3; meter multiplier setting 0.03; sensitivity 50.

phosphorylation by either higher plant chloroplasts or bacterial chromatophores. It is proposed that the factor(s) be named phosphodoxin, e.g., spinach phosphodoxin or *Rhodospirillum rubrum* phosphodoxin.

Methods.—*Rhodospirillum rubrum* chromatophores were prepared as described by Vernon and Ash,[6] and bacteriochlorophyll was determined by the method of Van Niel and Arnold.[7] The method of Anderson and Fuller[8] was used for the preparation of Chromatium chromatophores and the determination of bacteriochlorophyll. Spinach chloroplast fragments were prepared by isolation of intact chloroplasts in 0.35 *M* NaCl and 0.02 *M* Tris, pH 7.6, and then suspending the intact chloroplasts in 0.035 *M* NaCl and 0.002 *M* Tris pH 7.6. ATP was determined routinely as ATP^{32} by the method of Nielson and Lehninger[9] as modified by Avron.[10] In some experiments ATP was determined spectrophotometrically with glucose, hexokinase, NADP, and Zwischenferment. Reaction mixtures for assaying the photoproduction of ATP^{32} contained the following components in μmoles: Tris-HCl buffer, pH 7.5 or 7.8, 48; $MgCl_2$, 2; ADP, 1; Pi + P^{32} (containing 0.5–1 μc), 1; chlorophyll or bacteriochlorophyll, less than 30 μg; and water to a total volume of 1 ml. Reaction mixtures were illuminated laterally in 1 cm cuvettes at 2,500 ft-c at room temperature (19–20°C). When anaerobic conditions were employed, Warburg vessels were illuminated at 1,500 ft-c at 20°C. The vessels were flushed for 5 min with nitrogen or argon prior to illumination and throughout the illumination period to maintain anaerobic conditions. The vessels were shaken slowly throughout the gassing and illuminating procedure. Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer. Fluorescence and activation spectra were obtained with an Aminco-Bowman spectrophotofluorometer.

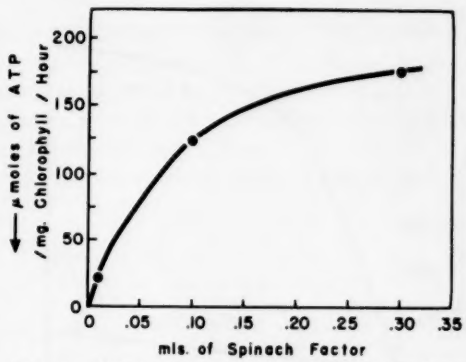

FIG. 3.—Effect of concentration of the active factor isolated from spinach chloroplasts on photophosphorylation of fragmented spinach chloroplasts.

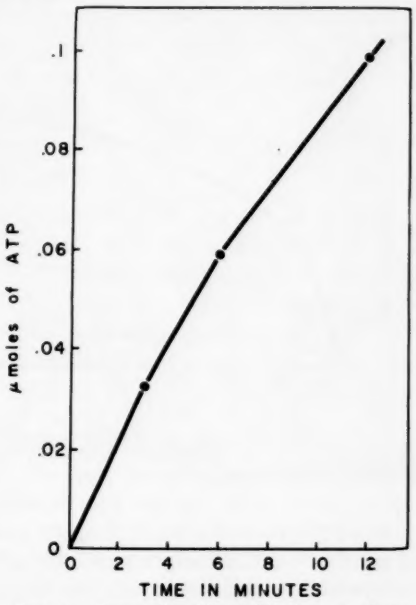

FIG. 4.—Proportionality with time of photophosphorylation of fragmented spinach chloroplasts in the presence of the factor.

Results.—Acetone powder of intact spinach chloroplasts was used as the source

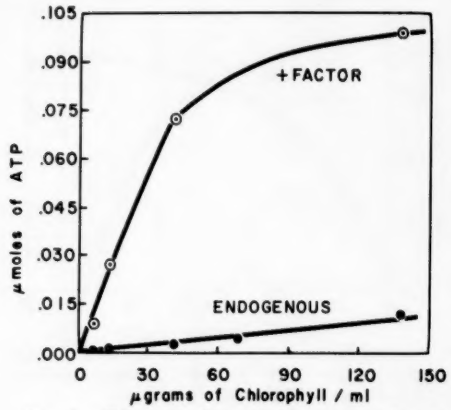

FIG. 5.—Effect of chlorophyll concentration on photophosphorylation of fragmented spinach chloroplasts.

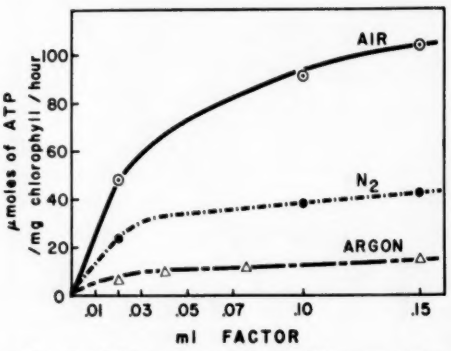

FIG. 6.—Effect of anaerobic conditions on photophosphorylation of fragmented spinach chloroplasts in the presence of the factor.

of the factor. The factor was isolated by suspending the acetone powder in water and heating. After cooling, the suspension was centrifuged and the pellet discarded. Following acetone fractionation (−20°C) of the supernatant, the preparation was chromatographed on Whatman 3MM paper using n-propanol: H_2O: 1% NH_4OH (3:1:2). The active fraction, which had an R_f of 0.47 in this solvent system, was eluted with water, and the eluted material was taken to dryness *in vacuo* and the residue extracted with ethanol. When the ethanol solution was allowed to sit at −20°C overnight, a precipitate formed, which was washed with ethanol and dissolved in water.

Absorption spectra of the active factor isolated from spinach chloroplasts are presented in the lower portion of Figure 1. There is a pronounced pH dependent shift in the absorption spectrum. The upper portion of Figure 1 shows the difference spectrum in 0.1 *N* HCl versus 0.1 *N* NaOH. This pH dependence is readily visible in that an acid solution of the factor is nearly colorless, whereas an alkaline solution is yellow.

Fluorescence and activation spectra of the active factor are given in Figure 2. A pronounced fluorescence peak was observed at 440 mμ, and this was activated maximally by irradiation at 358 mμ. The activation and fluorescence spectra (Fig. 2) indicate that the absorption maximum at 358 mμ observed in the alkaline absorption spectrum (Fig. 1) is connected with the fluorescence. The intensity of both the activation and fluorescence spectra (Fig. 2) is pH dependent in a fashion similar to the absorption spectrum (Fig. 1).

Many photosynthetic organisms were examined for a heat-stable, water-soluble factor which would catalyze photophosphorylation. The organisms tested and the rates of photochemical ATP production by fragmented spinach chloroplasts in the presence of the factor isolated from each organism are shown in Table 1. No significance is attached to the rate of ATP production observed in the presence of the factor isolated from each organism, except that a higher rate indicates that the factor has been further purified from that specific organism. Since all of these

activities were measured with fragmented spinach chloroplasts, it is evident that the organism from which the factor was isolated had little effect on the ability of the factor to catalyze photophosphorylation by chloroplasts. For example, factor(s) made from algae or a flagellate or photosynthetic bacteria are active with spinach chloroplasts. In addition, the factor made from spinach chloroplasts stimulates photophosphorylation by chromatophores from Chromatium and *Rhodospirillum rubrum*.[11]

TABLE 1

ORGANISMS CONTAINING A FACTOR WHICH STIMULATES PHOTOSYNTHETIC PHOSPHORYLATION

Source		μmoles of ATP/mg chlorophyll/hr*,†
Spinach chloroplasts		196
Spinach leaves		180
Phormidium luridum	Whole cells	49
Chlorococcum wimmeri	Whole cells	25
Chlamydomonas reinhardi	Whole cells, + strain	25
Tribonema aequale	Whole cells	27
Euglena gracilis	Whole cells	25
Chlorella pyrenoidosa	Whole cells	69
Rhodospirillum rubrum	Chromatophores	54
Rhodospirillum rubrum	Whole cells	50
Chromatium, strain D	Chromatophores	56
Chromatium, strain D	Whole cells	115

* The endogenous rate in these experiments varied from 0.5 to 3 μmoles of ATP formed per mg chlorophyll per hr.

† All preparations were assayed with spinach chloroplast fragments.

The effect of the concentration of factor isolated from spinach on photophosphorylation by fragmented spinach chloroplasts is shown in Figure 3. The reaction is linear with time for about 5 min (Fig. 4) and with chlorophyll concentration up to 40 μgrams of chlorophyll per ml (Fig. 5). Anaerobic conditions inhibited the reaction about 90 per cent with argon as the gaseous phase; with nitrogen only about 60 per cent inhibition was observed (Fig. 6). The possibility exists that the nitrogen contained a small quantity of oxygen which was sufficient to oxidize some component of the reaction mixture.

Rhodospirillum rubrum chromatophores support photophosphorylation in the absence of exogenous redox substances.[1] The data presented in Table 2 indicate that this endogenous photophosphorylation decays rapidly upon storage of the chromatophores. Furthermore, the factor isolated from *Rhodospirillum rubrum* stimulates ATP production by a fairly constant amount regardless of the endogenous rate of photophosphorylation (Table 2, last two columns). The time course

TABLE 2

STIMULATION BY FACTOR OF PHOTOSYNTHETIC PHOSPHORYLATION BY *Rhodospirillum rubrum* CHROMATOPHORES

Days after preparing chromatophores*	μmoles of ATP per mg bacteriochlorophyll per hr		
	Endogenous	Plus factor	Plus factor minus endogenous
0	81	193	112
3	68	206	138
4	15	146	131
5	17	139	122
6	28	168	140
10	15	126	111
20	21	149	128

* Chromatophores suspended in 0.1 *M* Tris, pH 7.8 plus 10% sucrose. Stored under vacuum and argon at 4°C.

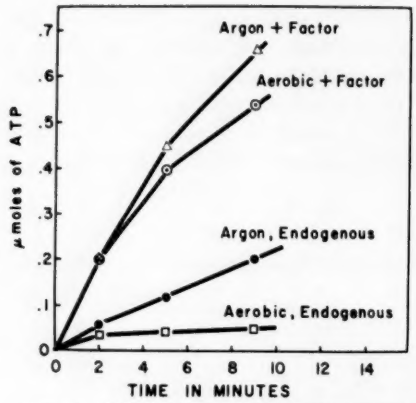

FIG. 7.—Effect of anaerobic conditions and time on photophosphorylation of *Rhodospirillum rubrum* chromatophores in the absence and presence of the factor isolated from *Rhodospirillum rubrum*.

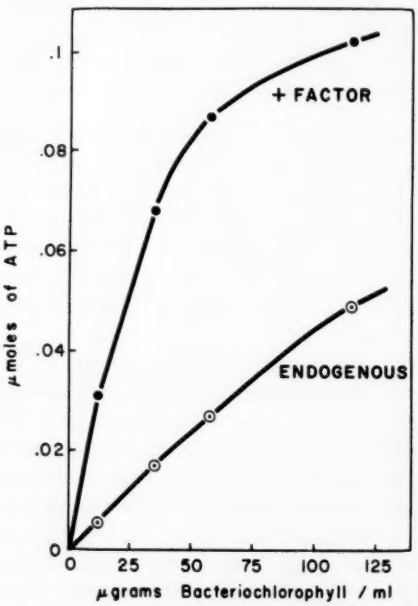

FIG. 8.—Effect of bacteriochlorophyll concentration on photophosphorylation by *Rhodospirillum rubrum* chromatophores.

for both the endogenous and the endogenous plus factor photophosphorylation is given in Figure 7. The effect of bacteriochlorophyll concentration is presented in Figure 8. In contrast to the photophosphorylation in the presence of the factor catalyzed by fragmented spinach chloroplasts under anaerobic conditions (Fig. 6), *Rhodospirillum rubrum* chromatophores in the presence of the factor are unaffected by anaerobic conditions after 2 min of illumination (Fig. 7). The endogenous photophosphorylation shows a marked stimulation under argon (Fig. 7, two lower curves), and this stimulation probably accounts for the slight increase under argon observed in the presence of the factor after 10 min of illumination (Fig. 7, two upper curves).

Discussion.—Several other naturally occurring redox "cofactors," namely, vitamins K_3 and K_5,[12] flavin mononucleotide,[13] "allagochrome,"[14] a "flavone-type" compound,[15] and photosynthetic pyridine nucleotide reductase,[16] have been shown to catalyze photophosphorylation. Therefore, a comparison of the properties of the factor reported herein with these other redox "cofactors" is pertinent. A comparison of the absorption and fluorescence spectra of flavins and vitamins K_3 and K_5[17, 18] with that of the factor (Figs. 1 and 2) indicates that the factor probably is not a flavin or vitamin K_3 or K_5. A further point of dissimilarity is that the factor reported in this paper is insensitive to ultraviolet irradiation even when irradiated for a period of 1 hr.

Habermann[14, 19] presented data which indicate that "allagochrome" has a component with an absorption peak at 673 mμ. This is not observed with the factor described here. Two further differences can be cited. First, "allagochrome" is not heat-stable, and secondly, it contains protein.[14, 19] In contrast, this new factor is heat-stable and devoid of protein. These characteristics also rule out photosynthetic pyridine nucleotide reductase.

Krogmann and Stiller[15] have reported that a "flavone-type" compound isolated from chloroplasts prepared in nonaqueous media catalyzed photophosphorylation. It was of interest, therefore, to determine if the factor was a flavone. The factor does not form lead, ferric, or ferrous salts characteristic of flavones and, furthermore, is insoluble in butanol and ethyl acetate. From a comparison of the absorption spectra of the factor (Fig. 1) with those given by Krogmann and Stiller, it is apparent that their compound and ours are different. It is also pertinent to point out that Chlorella contains the factor (Table 1) and that Chlorella does not contain flavones (Dr. R. Hill, personal communication). This comparison with the characteristics of other reported "cofactors" of photophosphorylation suggests that we have isolated a new, naturally occurring, heat-stable, water-soluble component of the photophosphorylation process.

Summary.—The isolation from photosynthetic organisms and the properties of a naturally occurring, water-soluble, heat-stable factor (phosphodoxin) which catalyzes photosynthetic phosphorylation either by spinach chloroplasts or bacterial chromatophores are presented. Phosphodoxin has been isolated from the following types of photosynthetic organisms: higher plant, algae, flagellate, and bacteria.

The authors are indebted to Mrs. Ferne Lubbers for the preparation of chromatophores, to Dr. T. E. Brown and Mrs. F. L. Richardson for the algal cultures, and to Dr. R. A. Lazzarini for the sample of Euglena.

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† Abbreviations used are: ADP and ATP, adenosine di- and triphosphate; NADP, nicotinamide adenine dinucleotide phosphate.

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*SIMILARITY OF DNAs ISOLATED FROM TUMOR-INDUCING VIRUSES OF HUMAN AND ANIMAL ORIGIN**

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Communicated by Edward A. Doisy, May 16, 1963

Preparations containing type 12[1,2] or 18[2] adenovirus have been recently reported by Trentin, Yabe, and Taylor[1] and Huebner, Rowe, and Lane[2] to induce tumor formation in newborn hamsters. These are the first "human" viruses reported to possess carcinogenic activity. In confirmation of these reports, we have isolated types 12 and 18 adenovirus in highly purified form and have induced tumor formation with as little as 0.2 μg of type 12 adenovirus.[3, 4] The induction of tumors with "pure" virus provides further convincing evidence that the virus itself, rather than some adventitious contaminant, is actually responsible for tumor induction. In the present communication, we wish to report on several properties of the DNAs isolated from tumorigenic types 12 and 18 adenoviruses and from nontumorigenic types 2 and 4 adenoviruses. A comparison of these properties suggests several provocative hypotheses.

Experimental Methods.—Virus: Seed cultures of the various adenoviruses were kindly given to us by Dr. R. J. Huebner. Types 2, 4, 12, and 18 adenoviruses were grown, isolated, and purified by minor modifications of the procedure described for type 2 adenovirus.[5, 6]

Viral DNA: Highly purified adenovirus (0.5 to 2 mg) was (1) incubated with crystalline papain, followed by (2) Duponol treatment and centrifugation in CsCl solution as described by Watson and Littlefield[7] for Shope papilloma virus (the salt precipitation step was deleted). Treatment with papain was required to separate viral DNA from a viral protein component. Yields of 50–80 per cent of the viral DNA were obtained. Examination of the viral DNA preparations in the analytical ultracentrifuge revealed one very homogeneous sedimenting component with $S_{20,w}$ of 30–32.[8]

Buoyant density of viral DNA: Viral DNA at 0.5–2 μg per ml in CsCl solution (density 1.71, in 0.01 *M* Tris buffer, pH 8.1) was centrifuged in the Spinco E analytical ultracentrifuge[9] together with a density reference of 1 μg/ml of *Ps. aeruginosa* DNA (kindly given to us by Dr. N. Sueoka). Ultraviolet absorption photographs of the DNA bands were taken after 20 hr at 44,770 rpm and traced with a Spinco Analytrol. Densities were calculated[10] by comparison with the *Ps. aeruginosa* DNA standard. The latter was assigned a buoyant density of 1.727 to facilitate comparison with the DNA buoyant densities compiled by Schildkraut, Marmur, and Doty.[11]

Denaturation temperature (T_m): The T_m value for each viral DNA was determined in saline citrate (0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7.4) with a thermostatically controlled Beckman DU spectrophotometer as described by Marmur and Doty.[12]

Results and Discussion.—Table 1 summarizes the buoyant density and T_m values that we have found for the DNAs isolated from types 2, 4, 12, and 18 adenoviruses. Also included are the corresponding values for the DNAs of the two

tumor-inducing animal viruses thus far reported: polyoma virus[13, 14] and Shope papilloma virus.[7] The base composition listed for each double-stranded viral DNA (expressed as per cent guanine-cytosine) was either determined by direct base analysis or calculated from its buoyant density[11] or T_m.[12] The base composition calculated from the buoyant density and T_m are in good agreement with each other and with the results of direct analysis (where carried out).

TABLE 1

SIMILARITY IN BASE COMPOSITION OF DNA-CONTAINING MAMMALIAN TUMOR VIRUSES

Virus	Tumor induction	Buoyant density	Denaturation temperature (T_m)	% Guanine-cytosine
Adenovirus type 2	No	1.716	92.5°	56*
Adenovirus type 4	No	1.718	92.5°	57*
Adenovirus type 12	Yes	1.708	89.0°	48†
Adenovirus type 18	Yes	1.709	89.0°	49†
Polyoma virus	Yes	1.709[13]	89.0°[14]	48†
Shope papilloma virus‡	Yes	1.711[7]	89.5°[7]	49§

* Base composition by direct chemical analysis.[5, 6]
† Calculated from buoyant density[11] and denaturation temperature.[12]
‡ The buoyant density reported for the DNA of Shope papilloma virus[7] (1.714) was changed to 1.711 for this comparison to compensate for the different buoyant density values assigned the reference DNA standard.
§ Base composition by direct chemical analysis.[7]

A comparison of the base compositions of these viruses reveals several interesting and startling features. First, it should be noted that the DNAs of the nontumorigenic types 2 and 4 adenoviruses have 56–57 per cent guanine-cytosine, while the DNAs of the tumorigenic types 12 and 18 adenoviruses have 48–49 per cent guanine-cytosine. Thus, both types 12 and 18 adenovirus DNA have base compositions quite different from that of types 2 and 4 and closer to that of cell DNA (mammalian cell DNA has 42–44 per cent guanine-cytosine).[15] It is possible that tumorigenic adenoviruses evolved from nontumorigenic adenoviruses by deletion of a piece of DNA rich in guanine-cytosine. This possibility is consistent with data showing that the DNA of type 12 adenovirus has a lower molecular weight than that of type 2 adenovirus.[8] An alternate possibility, suggested by the large differences in base composition, is that the tumorigenic and nontumorigenic adenoviruses are quite unrelated genetically. Further evidence is needed to settle this point.

Perhaps of much greater interest is the fact that the four tumor-inducing DNA-containing mammalian viruses, namely, adenovirus type 12, adenovirus type 18, polyoma virus, and Shope papilloma virus, have very similar buoyant densities, T_m values, and therefore base compositions. This similarity is conceivably fortuitous. Alternatively, it may reflect a common evolutionary origin for these viruses. A third and more exciting and speculative possibility is that virus tumorigenicity may depend upon its DNA having regions homologous in structure to a segment of host cell DNA. Thus, it may be hypothesized that a region of cell DNA of approximately one millionth that of the whole genome has a base composition similar to that of the tumor viruses. (There is approximately one million times as much DNA per cell as per adenovirus particle.) Tumor virus DNA may be able to replace or combine with this area of host cell DNA. This process would subvert the function of this region of host cell DNA whose role may involve the control of cell division.

Summary.—Tumorigenic and nontumorigenic types of human adenovirus were isolated; the buoyant density, denaturation temperature, and base composition of their DNAs were determined. The DNAs of nontumorigenic types 2 and 4 adenoviruses contained 56–57 per cent guanine-cytosine, while those of tumorigenic types 12 and 18 adenoviruses were surprisingly different and contained 48–49 per cent guanine-cytosine. A striking similarity between the DNAs of the tumor-inducing viruses of human origin, i.e., adenovirus types 12 and 18, and those of animal origin, polyoma virus and Shope papilloma virus, is noted: all four tumor viruses have very similar buoyant densities, denaturation temperatures, and base compositions. The possible implications for the evolution of tumor-inducing viruses and for the mechanisms of virus-induced tumorigenicity are briefly discussed.

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† Research Career Awardee of the National Institutes of Health.

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*DIALYSIS STUDIES, VIII. THE BEHAVIOR OF SOLUTES WHICH ASSOCIATE**

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The recent rapidly expanding interest in hemoglobin stems in part from the fact that, in addition to its interesting function, it is a readily available protein of the largest size thus far to yield to the detailed structural investigations of the chemist. It has become a useful model for many different types of biochemical investigations. These in turn have often contributed to a better understanding of the over-all structure of the molecule.

A study of hemoglobin was undertaken in this laboratory several years ago for several reasons. Apart from the challenge presented by an attempt to elucidate the sequential arrangement of the amino acids, it offered an excellent model with

which to improve the laboratory tools needed for such a study. Moreover, it was considered to be a complex of four protein chains[1] arranged in a highly specific way,[2] and thus of great interest as a model in the membrane diffusion[3] and ultracentrifugal[4] studies under way in this laboratory. This paper will deal with an aspect of the hemoglobin study chiefly concerned with association-dissociation phenomena and the possible conformational changes which contribute to the unique chemistry of this substance.

Several years ago it was noted that with the thin film dialysis technique a cellophane membrane could be prepared by a stretching process which would readily pass ovalbumin (M.W. 44,000) but totally exclude serum albumin (66,000). This membrane also permitted diffusion of a crude sample of bovine hemoglobin at an acid pH, but since hemoglobin was known to dissociate in acid, this behavior was considered entirely normal. Indeed, Matsuda, Schroeder, and Martin[5] separated the alpha chains from the gamma chains in fetal globin by a simple dialysis procedure.

Further investigation, however, has revealed that diffusion of hemoglobin will take place rather rapidly at low ionic strength and even at a neutral pH through a membrane which will virtually exclude a molecule of 66,000. It does this under the conditions for which the tetramer is considered the most stable, and where the ultracentrifuge has shown the molecular species of size 66,000 to be the only one present, or at least to be by far the most predominant species present.

This apparent paradox can be very significant as far as the chemistry of hemoglobin is concerned, and in addition can contribute to a very interesting aspect of the study of large molecules by the dialysis method. If indeed a small fraction of the dimer (or even monomer) is present but the rate of association and dissociation is relatively rapid as compared to the dialysis rate, then a certain amount of diffusion through the membrane could take place. The question of the over-all rate of diffusion to be expected, however, is not so simple. On the basis of experience with a number of other solutes which the ultracentrifuge has shown to be strongly associated depending on the solvent, e.g., subtilin, ACTH, insulin, and others, a rate of diffusion equal to or not much slower than expected from the monomer has been found. Apparently, this approach to an estimation of the minimal molecular size is less affected by the uncertainties arising from readily reversible association than is the sedimentation method. Perhaps this stems from the fact that the basis of the dialysis method is a kinetic one which reflects preferentially the diffusional activity of the smallest particle.

With homogeneous solutes which do not associate, the rate of diffusion through the membrane is directly proportional to the concentration gradient across the membrane. It would therefore appear that in the case of a polymer which, as such, cannot diffuse through the membrane, the concentration of only the monomer would be the controlling concentration. However, since with hemoglobin and several other associating solutes this does not seem to hold, an explanation of the result is clearly required. One explanation could be that the surfaces throughout the membrane favor dissociation.

Perhaps a related but more likely explanation stems from the probability that the monomer reassociates after entering the pores of the membrane. If so, the physical nature of the membrane becomes highly important, as well as the fact that

it actually is very thick in relation to the molecular diameters of the diffusing molecules, of the order of 1,000 to 10,000 times their diameters. Therefore, with a nonassociating solute that has no tendency to be adsorbed on the surface of the membrane, there will be a rapid fall in solute concentration across the membrane with the maximum concentration essentially the same immediately within the entering boundary as the concentration of the retentate solution. But in the case of an associating solute with the monomer always capable of rapid reassociation, the concentration of monomer plus polymer within the entering boundary will be proportional to the total concentration of monomer plus polymer also on the retentate side of the membrane rather than to the concentration of monomer alone. If such a mechanism should hold, the rate of diffusion through the membrane would then tend to be proportional to the total concentration rather than to that of the monomer alone and the over-all diffusion rate would be near that expected from a nonassociating solute the size of the monomer. Even a straight line escape plot could result, depending on the type of association equilibrium characteristic of the solute.

On the other hand, if the rate of association or dissociation should be sufficiently slow, this would probably be reflected in a reduced rate of dialysis and could also lead to a deviation from a straight line escape plot. Therefore, the linearity of the escape plot[6] over a wide range of concentration can be of considerable interest where associating solutes are under study.

Two general types of deviation might be expected. The ideal solute homogeneous with respect to size should give the straight line plot of *A* (Fig. 1). Experimentally, preparations of many different kinds of solutes of a broad range of sizes have been shown to conform with surprising precision. If not, further purification is indicated.[3] Certain solutes, however, have consistently shown a deviation in spite of extensive fractionation attempts and other evidence of their homogeneity. For instance, a highly purified sample of insulin gave a negative deviation[7] of the type *B* of Figure 1, while ribonuclease showed a positive deviation of the type *C*. In the case of insulin, good evidence was presented that the negative deviation noted was caused by dissociation of the dimer,[7] the main species present at the beginning or highest concentration. More recently, convincing evidence[8] has been found that the positive deviation noted with ribonuclease can result from conformational isomers, slowly interconvertible under the right conditions, and, to a lesser degree, from polymers not readily dissociated.

The finding of the influence of subtle changes of shape in membrane diffusion with ribonuclease had earlier been indicated.[6] This also can be of importance to the interpretations of the type of curve obtained with associating solutes. Undoubt-

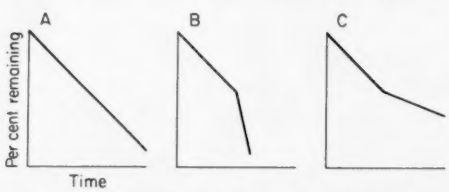

FIG. 1.—Hypothetical escape plots (the ordinate is in logarithms).

edly, the association-dissociation equilibrium will, to some degree, involve a change in conformation of the monomer. Thus, it would not be surprising to find a homogeneous associating solute which would give any of the three types of escape curves shown in Figure 1.

With these possibilities in mind, a number of experiments were made with carbonmonoxyhemoglobin. Quite apart from the association-dissociation problem, it seemed of interest to investigate the possibility of detecting a small shift in size of the hemoglobin molecule during loss or gain of oxygen. Unfortunately, with the present membranes oxyhemoglobin has proved entirely unsuitable for a careful study due to the fact that it was too strongly adsorbed to or in the membrane. Interestingly enough, carbonmonoxyhemoglobin did not show this property and behaved well in the experiments.

Experimental.—The dialysis cell used in these experiments was of the type previously described.[9] The porosity of the membrane was adjusted to a suitable range by treatment[9] of 20/32 Visking cellophane casing with a solution of 64% $ZnCl_2$ for 6 min at 25°. All the measurements reported in this paper were made at 25° in the same cell and membrane. They are therefore strictly comparable. They were made in an atmosphere of carbon monoxide by placing the cell assembly in a filter flask of appropriate size which was closed with a rubber stopper after flushing out the air with a stream of carbon monoxide. Withdrawal of fractions was accomplished by opening the flask, after which it was again flushed with carbon monoxide. The mechanical stirring feature more recently used in thin film dialysis[9] was omitted. It was known from the earlier work that the periodic withdrawal of samples seemed to afford sufficient stirring for reproducible comparisons, even though the rate was uniformly slower. The concentrations of the dialysate fractions were determined by measuring their absorbance at 415 mμ in a Beckman spectrophotometer.

The hemoglobin used for this study was a hemolysate prepared as previously described[10] from the freshly drawn blood of one individual (Guidotti). Previous analysis of this hemoglobin had shown it to contain approximately 96% hemoglobin A. For this particular study it appeared advisable to avoid any type of fractionation. Cytochrome C was purchased from Sigma Chemical. Myoglobin was purchased from Mann Research Co. Chymotrypsinogen and pepsinogen were purchased from Worthington. The bovine serum albumin was a sample obtained from Armour.

The membrane was calibrated under the various conditions used with a series of proteins as shown by the data in Table 1.

Results and Discussion.—Comparative data for carbonmonoxyhemoglobin are shown in the escape plots of Figure 2. From these results it can be seen that

TABLE 1

THE HALF ESCAPE TIMES OF THE SOLUTES USED TO CALIBRATE THE MEMBRANE

Solute	M. W.	Conc. g %	Half escape times in hr; solvent: 0.2 *M* NaAc pH 7	0.2 *M* NaAc 2 *M* NaCl pH 7	0.2 *M* NaAc 0.2 *M* HAc pH 4.7
Ferricytochrome C	12,000	0.4	0.95	1.1	—
Myoglobin	17,816	0.4	1.25	1.4	1.6
Chymotrypsinogen	25,100	0.7	3.2	3.8	3.0
Pepsinogen	40,000	0.5	8.5	—	—
Albumin (bovine plasma)	66,000	0.8	18–20	18–20	22

the protein diffuses through the membrane at a pH of 4.7 a little more slowly than does chymotrypsinogen. This rate is entirely consistent with the size expected from the hemoglobin molecule, if it is dissociated into two parts each with a molecular weight of 33,000. Such a dissociation below pH 5 has now become a well-accepted property of the larger molecule.

When the pH was adjusted to 7 in potassium acetate buffer, the rate of diffusion of the hemoglobin was considerably less (Fig. 2), and corresponded more to the rate expected from a molecule of about the size or slightly larger than pepsinogen, molecular weight of 40,000. However, the rate found was definitely too rapid for a globular protein in the range of 66,000 molecular weight.

It seemed of interest that the escape plots of hemoglobin in Figure 2 were surprisingly linear over the concentration range covered. Concentration dependence would be expected if association were involved. However, the range covered in a single experiment involves a change in concentration no greater than from 2–3-fold, and this might not be sufficient to reveal concentration dependence, unless a particularly striking deviation were involved. Therefore, a series of comparative runs were made to cover a wider range of concentration. The results of these experiments in terms of 50 per cent escape times are given in Table 2. These data

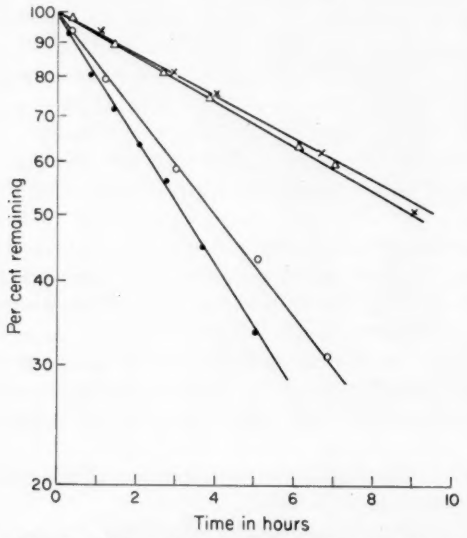

FIG. 2.—Escape plots at 25°. ●—● = chymotrypsinogen at pH 4.7 (0.2 *M* NaAc); ○—○ hemoglobin at pH 4.7 (0.2 *M* KAc); △—△ = hemoglobin at pH 7.0 (0.2 *M* KAc); ×—× = hemoglobin at pH 6.8 (0.2 *M* KAc + 2 *M* NaCl).

show clearly that at pH 7.1 in phosphate buffer at two ionic strengths dialysis takes place more rapidly as the retentate solution becomes more dilute, but apparently so only below an initial concentration level of 0.3 per cent. This shift is less marked at a pH of 4.71. A faster rate of diffusion on dilution usually would be expected from the equilibrium of a dissociating solute. The dilution effect is shown graph-

TABLE 2

HALF ESCAPE TIMES OF CARBONMONOXYHEMOGLOBIN AT 25° IN DIFFERENT SOLVENTS AND AT VARIOUS CONCENTRATIONS

Initial conc. %	0.2 *M* KAc 0.2 *M* HAc pH 4.71	0.2 *M* KAc 0.2 *M* HAc pH 4.7 2 *M* NaCl	0.2 *M* PO_4 pH 7.1	0.2 *M* PO_4 pH 7.1 2 *M* NaCl	0.2 *M* KAc pH 6.8 2 *M* NaCl
3.78	6.5		8.4	9.5	
0.77				9.3	
0.35	3.8				
0.31		9.2	7.8	8.5	9.5
0.25		8.4			
0.11		6.2	6.2	6.8	5.7
0.07	3.0	*	4.5	5.8	
0.038	1.75		2.75	5.2	3.5

* ppt. out.

ically in Figure 3 for three solvent conditions. The half escape times expected (from Table 1) for monomer, dimer, and tetramer are 1.5, 5, and 18–20 hr, respectively. The higher ionic strength favors association. This could be taken to indicate that the forces favoring association are not electrostatic in nature.

Escape rates were also determined for a series of pH values ranging from 3.2 to 11.54 in phosphate buffer. Figure 4 shows a plot of the 50 per cent escape times found against pH. Since previous experience with other proteins had shown that phosphate ion itself had a specific effect different from other buffers, part of the pH range was also studied in acetate buffer. These results are also presented in Figure 4.

From data obtained through sedimentation studies at different pH values Hasserodt and Vinograd[11] postulated the dissociation of human adult hemoglobin into dimers at pH values less than 5 or greater than 10. The data of Figure 4 are completely consistent with this behavior. In addition, it is possible to draw other

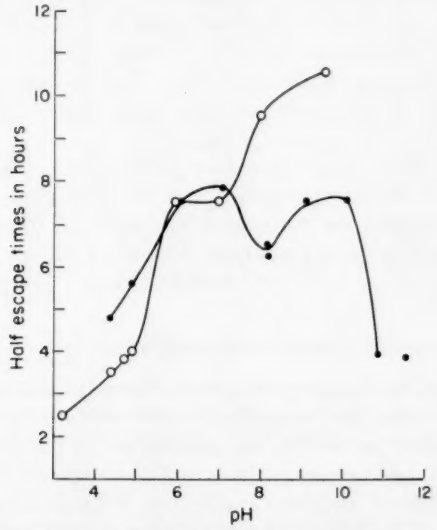

FIG. 3.—Effect of pH on escape rate. ●—● in phosphate buffer; O—O in acetate buffer.

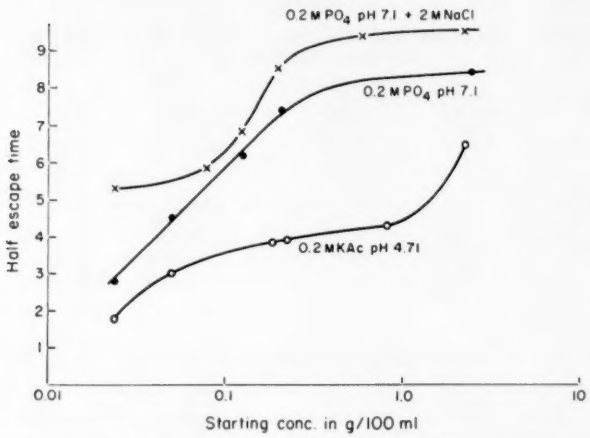

FIG. 4.—Effect of concentration on escape rate of hemoglobin.

inferences. Ion binding probably also plays a role. This may account for the minimum in the curve noted at pH 8.2, and could in part account for the strikingly different behavior noted in acetate. It is interesting that the results in acetate at low pH indicate partial dissociation beyond the dimer stage to the monomeric single chain. This possibility seems all the more likely since all proteins we have studied thus far which do not associate begin to show a reduced rate of diffusion at low pH rather than an increased rate, apparently due to denaturation or expansion of the molecule.

A series of experiments on the effect of a higher ionic strength on the association equilibria of hemoglobin have been reported.[12,13] It was of interest therefore to repeat certain of the experiments described above in 2 *M* NaCl. The data from these experiments are included in Table 2. Perhaps the most striking result from the salt effect is that in acetate buffer at pH 4.7. Here the rate of diffusion is similar to that at pH 7, a result which could indicate that the higher ionic strength inhibits the dissociation otherwise taking place at this pH.

The experiments reported in this paper are pertinent to a considerable volume of recent literature[11, 14] on the dissociation and recombination of the subunits of hemoglobin. They support the proposal of Vinograd and Hutchinson[15] that there is first a symmetrical dissociation to dimers and also a further partial dissociation of the dimers at low pH to individual peptide chains. The over-all equilibrium can be written as follows:

$$\alpha_2\beta_2 \rightleftarrows 2(\alpha\beta) \rightleftarrows 2\alpha + 2\beta$$

It is not the purpose of this paper to discuss the many fascinating studies that have been made on dissociation and recombination of mixed hemoglobins. These can, however, be explained on the basis of the $\alpha\beta$ dimer as the one preferred over the α_2 and β_2 dimers, where the environment is favorable to dimer formation, of tetramer formation by association of $\alpha\beta$ dimers at pH 7 but still with dimers present, and of monomer formation at low pH or low concentration where the equilibrium is shifted in the other direction. The interpretation of the results must also take account of

the fact that the equilibria are influenced by the ionic strength of the solution in addition to the pH and even by the type of ions present in solution to furnish the ionic strength. The concentration of the hemoglobin below a certain critical level can play a role as might be expected.

From the standpoint of the behavior of associating solutes in thin film dialysis, the hemoglobin study is particularly interesting because it concerns a solute which associates in a specific way to limited sizes, e.g., dimers and tetramers. Other types of associating solutes have shown their own characteristic behavior. Thus, insulin, ACTH, and glucagon at appropriate concentration levels gave escape curves of the type *B* in Figure 1. The majority of proteins and peptides studied have shown a reduced rate of diffusion when a salt or urea was added to the solution as compared to the rate in distilled water or 0.01 *N* acetic acid. Associating solutes show abnormal behavior in this comparison. Thus, tyrocidine B, a cyclic decapeptide which shows strong association in the ultracentrifuge, shows a faster diffusion rate in 6 *M* urea than in 0.01 *N* acetic acid.

Subtilin, a cyclic peptide of molecular weight 3,200,[16] shows strong reversible association to high molecular weight material in the ultracentrifuge. However, in 0.01 *N* acetic acid with the dialysis technique it gives a straight line escape plot and at a rate consistent with the monomer as compared to other solutes of this molecular weight range. In a 0.2 *M* ammonium acetate buffer identical to that used in the ultracentrifugal studies the rate, however, was retarded by a factor of 10, and strong deviation from straight line behavior was observed. The deviation was of type *C* in Figure 1. Addition of urea (to 6 *M*) in this case slowed the rate even further by a factor of 2.

Glucagon (29 amino acid residues[17]) shows a faster escape rate in urea than in 0.01 *N* acetic acid and a change to straight line behavior. ACTH (39 amino acid residues) has been well documented[18] with respect to its association behavior in the ultracentrifuge. It dialyzes at a rate consistent with a monomer in 0.01 *N* acetic acid in spite of the association. However, in 0.2 ammonium acetate buffer the dialysis rate drops to only one tenth that in 0.01 *N* acetic acid. Since ACTH studied in this buffer in the ultracentrifuge was found to be essentially monomer, a very large conformational shift for the change of solvent environment of from 0.01 *N* acetic acid to 0.2 *N* ammonium acetate buffer at pH 4.1 seems to be indicated. In 6 *M* urea the rate is one third that in the 0.01 acetic acid.

These results will be reported in full elsewhere. Obviously, association phenomenon with peptides offers a rich field for study by a combination of the ultracentrifuge and thin film dialysis.

We are indebted to Dr. D. Yphantis for the ultracentrifuge studies.

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*IDENTIFICATION OF PEPTIDES SYNTHESIZED BY THE CELL-FREE E. COLI SYSTEM WITH POLYNUCLEOTIDE MESSENGERS**

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Characterization of the products formed by cell-free systems of protein synthesis as a result of the incorporation of amino acids into acid-insoluble products, promoted by synthetic polyribonucleotides, has lagged behind the use of these compounds in studies on the genetic code.[1, 2] Nirenberg and Matthaei[3] partially characterized the product of phenylalanine incorporation by the *Escherichia coli* system, in the presence of poly U, as poly L-phenylalanine. This characterization was based on comparison with an authentic sample of the chemically synthesized compound as regards solubility and susceptibility to acid hydrolysis. However, the insolubility of the product in aqueous solvents precludes identification through cleavage with proteolytic enzymes. This is also true of polypeptides made with copolynucleotide messengers rich in uridylic acid. The insolubility of these products also interferes with the determination of their chain length, or the identification of the C- and N-terminal amino acids, by conventional methods of end-group assay.[4]

The finding[5] that poly A directs the synthesis of polylysine which, although insoluble in tungstic acid, is soluble in trichloroacetic acid and in aqueous solvents, opened the way for enzymatic and chemical studies of the products synthesized by cell-free systems with poly A or with adenylic acid-rich copolynucleotide messengers. Like chemically synthesized polylysine, the product of the poly A-promoted lysine incorporation was susceptible to hydrolysis by trypsin, but resistant to the action of chymotrypsin.[5] Further work, to be reported in this paper, has shown that the product of lysine incorporation with poly A as messenger yields di- and trilysine on tryptic digestion. This conclusively identifies this product as poly-L-lysine.

All of the eight triplets occurring in AU copolymers have been assigned as follows:[5] AAA, lysine; 2AlU, asparagine, isoleucine, lysine; 1A2U, isoleucine, leucine, tyrosine; UUU, phenylalanine. Thus, AU copolymers promote the incorporation of the six amino acids, asparagine, isoleucine, leucine, lysine, phenylalanine, and tyrosine. As reported in this paper, high-voltage electrophoresis of tryptic digests of the products formed, in the presence of poly AU (5:1), with either (*a*) lysine-C^{14}, (*b*) isoleucine-C^{14}, (*c*) lysine-C^{14} + isoleucine-C^{14}, or (*d*) lysine-C^{14} + asparagine-C^{14}, and the remaining four or five amino acids without radioactive label, yields a series of radioactive peptide peaks. The major peaks are six in number when lysine-C^{14} is present in the incubation mixture, but only four when the C^{14} label is in amino acids other than lysine. Peaks 5 and 6 move in the position of di- and trilysine. Upon acid hydrolysis, peaks 1 through 4 all yield radioactive isoleucine + lysine and asparagine + lysine in experiments (*c*) and (*d*), respectively. These results show that synthetic copolynucleotide messengers give rise to copolypeptides in the *E. coli* system.

Methods.—Composition of reaction mixtures: All reaction mixtures contained per ml (in μmoles unless otherwise stated) Tris-HCl buffer, pH 7.9, 37; Lubrol, 1.8 mg; KCl, 55; $MgCl_2$, 13; mercaptoethylamine, 11; ATP, 0.9; GTP, 0.22; creatine phosphate, 12; creatine kinase, 44 μg; *E. coli* transfer RNA, 3 mg; *E. coli* ribosomes, 2 mg protein; and *E. coli* supernatant, 4 mg protein. In addition, in experiments with poly A the samples contained polymer, 40 μg, and lysine C^{14}, (7 μc/μmole), 0.2. In experiments with poly AU (5:1) they contained polymer, 80 μg, and asparagine, isoleucine, leucine, lysine, phenylalanine, and tyrosine, each 0.05. In this case one or more amino acids were labeled with C^{14} and were used at specific radioactivities between 50 and 100 μc/μmole. Incubation was for 30 min at 37°.

Isolation of lysine-rich polypeptides by differential precipitation: One advantage of dealing with polylysine or lysine-rich polypeptides is that, due to their solubility in trichloroacetic acid,[6] they can be largely separated from proteins in the reaction mixture including C^{14}-labeled, trichloroacetic acid-insoluble material which is always formed by the system in small amounts, whether in the presence or absence of synthetic polynucleotides, probably as a result of the presence of residual messenger RNA in the *E. coli* fractions. This separation was accomplished as follows. An equal volume of 10% trichloroacetic acid was added to the reaction mixture after incubation. The precipitate was collected by centrifugation, washed three times with 2 ml of 5% trichloroacetic acid, each time with heating for 15 min at 90°, and dissolved in 0.5 *N* KOH. This fraction (Fraction A) contained some radioactive protein. After digestion of this material with trypsin followed by high-voltage paper electrophoresis, there was little or no migration of radioactivity from the origin. To the supernatant, which was combined with washings of the trichloroacetic acid precipitate, was added 1.0 mg of carrier polylysine followed by sodium tungstate to a final concentration of 0.25%. The precipitate was recovered by centrifugation, washed three times with a solution of 0.25% sodium tungstate in 5% trichloroacetic acid, adjusted to pH 2.0, each time with heating for 15 min at 90°, and finally taken up in 1.6 *N* ammonium hydroxide. Insoluble material having little radioactivity was removed by centrifugation and discarded. The clear ammonium hydroxide solution (Fraction B) contains the bulk of the polylysine or lysine-rich polypeptides. In an experiment with poly AU (5:1) (isoleucine-C^{14}, lysine-C^{14}, cold asparagine, leucine, phenylalanine, and tyrosine), 25,000 cpm were recovered in Fraction A and 477,000 cpm in Fraction B.

Trypsin digestion and separation of peptides: Fraction B was evaporated to dryness in a flash evaporator and the residue taken up in about 2 ml of water. To the suspension was added 0.5 ml of 10% ammonium bicarbonate, 1.0 mg of carrier polylysine, and crystalline trypsin (0.05 mg in experiments with poly A, 0.2 mg in experiments with poly AU). The volume was made up with water to 5.0 ml and the mixture incubated for 16 hr at 37°. The tryptic digest was evaporated to dryness and dissolved in a few ml of water. The evaporation and solution were repeated several times to ensure complete removal of the ammonium bicarbonate. The final residue was dissolved in as little water as possible, and this solution subjected to either paper chromatography or paper electrophoresis.

In experiments with poly A, the products of tryptic digestion were separated by descending chromatography for 48–72 hr in *n*-butanol: acetic acid: water: pyridine (30: 6: 24: 20 v/v) according to Waley and Watson.[7] Under these conditions, lysine, dilysine, and trilysine moved about 15, 9, and 6 cm, respectively, from the origin in 48 hr. In experiments with poly AU, separation of the products of tryptic digestion was accomplished by high-voltage paper electrophoresis.[8] In most cases electrophoresis was conducted for 2.5 hr at 4,000 volts in pyridine: acetate: water (10:1:69 v/v, pH 6.0) and the samples were banded near the anode. Under these conditions, lysine, dilysine, and trilysine moved 57, 66, and 71 cm, respectively, toward the cathode. All neutral amino acids migrated 8 cm in the same direction. Authentic samples of amino acids and a tryptic digest of authentic poly-L-lysine, containing di- and trilysine and some tetralysine, were used as markers. The spots were located with ninhydrin spray, and radioactivity was detected with use of a strip counter (Nuclear-Chicago Actigraph) supplemented occasionally by autoradiography.

Other methods: In experiments with poly AU, following separation of the tryptic peptides by paper electrophoresis, paper strips corresponding to radioactive peaks were cut out, shredded into small pieces, and eluted with 10 ml of water overnight. Approximately 60% of the radioactivity was recovered in this way. After decanting the fluid, the paper residue was washed twice with 3–5 ml of water, and the washings were combined with the eluate. The solution was dried by flash evaporation, the residue dissolved in 5.7 *N* hydrochloric acid of constant boiling point, and the peptides were hydrolyzed in a sealed tube for 17 hr at 106°. The hydrolysate was evaporated to dryness, redissolved in water, and again evaporated. This procedure was repeated several times for complete removal of the hydrochloric acid. The residue was finally dissolved in a small amount of water and spotted on Whatman No. 1 paper for chromatographic separation of the amino acids. For separation of isoleucine and lysine the chromatogram was developed overnight with *n*-butanol: acetic acid: water (4:1:5 v/v);[9] R_F isoleucine, 0.64; R_F lysine, 0.10. For separation of aspartic acid and lysine (asparagine being converted to aspartic acid by acid hydrolysis), the chromatogram was developed overnight with phenol-ammonia;[10] R_F aspartic acid, 0.12; R_F lysine, 0.80. The radioactivity on the paper strips was scanned with an actigraph.

Preparations.—E. coli ribosomes, supernatant, and transfer RNA were prepared as previously described.[11] We are indebted to Dr. A. J. Wahba for some of these fractions. Data on the poly A and poly AU (5:1) used in this work have been reported.[5] A mixture of di-, tri-, and tetralysine was prepared as follows. 10 mg of poly-L-lysine (mol wt, 200,000; Mann Research Laboratories, Inc., New York) and 0.2 mg of trypsin, in 1.0 ml of 0.1 *M* potassium phosphate buffer, pH 8.0, were incubated for 16 hr at 37°. Of this digest, 0.005–0.01 ml aliquots were spotted on paper to provide di-, tri-, and tetralysine markers in paper chromatography or electrophoresis. On chromatography in the *n*-butanol: acetic acid: water: pyridine solvent, three spots were obtained, the R_F values of which, relative to that of lysine, agreed reasonably well with those reported by Waley and Watson[12] for the authentic peptides. The major spots were di- and trilysine but a tetralysine spot was detectable. No lysine was detected.

Asparagine-C^{14} of high specific radioactivity (82 $\mu c/\mu mole$) was prepared by ammonolysis of aspartyl-C^{14} phosphate. This was obtained by enzymatic phosphorylation of aspartic acid-C^{14} with ATP, a reaction catalyzed by aspartate kinase (E.C. 2.7.2.4).[13] Yeast aspartate kinase (specific activity, 0.08 μmole/min/mg protein at 30°) was kindly provided by Drs. C. Gilvarg and Y. Yugari of this department. The reaction mixture contained per ml (in μmoles unless otherwise stated) Tris-HCl buffer, pH 8.0, 100; KCl, 100; $MgCl_2$, 10; ATP, 10; phosphoenolpyruvate, 10; DPNH, 2.5; aspartic acid-C^{14} (specific radioactivity, 82 $\mu c/\mu$mole, New England Nuclear Corporation, Boston, Mass.), 1.5; aspartate kinase, 1.7 mg protein; and an excess of crystalline pyruvate kinase (E.C. 2.7.1.40)[13] and lactate dehydrogenase (E.C. 1.1.1.27).[13] Incubation was for 30 min at 30°. The ADP formed by the transphosphorylation between ATP and aspartic acid is rephosphorylated by transfer of phosphate from phosphoenolpyruvate, catalyzed by pyruvate kinase, thus displacing the equilibrium in favor of aspartyl phosphate formation. The reaction was followed spectrophotometrically by the decrease in absorbancy at wavelength 340 mμ due to the lactate dehydrogenase-catalyzed oxidation of DPNH by the pyruvate formed from phosphoenolpyruvate.

After incubation, the reaction mixture was added dropwise into 50 ml of 28% ammonia and kept at room temperature for 30 min. Excess ammonia was removed by evaporation to dryness

in vacuo, the residue was dissolved in 10 ml of water, and the solution adjusted to pH 9.0. The asparagine-C^{14} was separated from excess aspartic acid-C^{14} by ion-exchange chromatography. The solution was applied to a Dowex-1 (acetate) column (1 × 13 cm); the column was then washed with water, and the asparagine eluted with 0.02 *N* acetic acid as a sharp, symmetric peak. The aspartic acid-C^{14} could be subsequently recovered by elution with 1.0 *N* hydrochloric acid. The fractions containing the asparagine-C^{14} were pooled and lyophilized. The compound migrated as a single radioactive spot on paper chromatography in methanol:pyridine:water (8:0.4:2 v/v).[14] Based on the aspartic acid-C^{14} used, the yield of asparagine-C^{14} was 33%.

Other C^{14}-labeled amino acids of high specific radioactivity (0.85–1.0 mc/mg) were purchased from Schwarz BioResearch, Inc., Orangeburg, New York. Crystalline pyruvate kinase and lactate dehydrogenase were purchased from C. F. Boehringer and Sons, Mannheim, Germany. The source of other preparations and materials was as stated in previous publications from this laboratory.[5, 11]

Results.—Experiments with poly A: As shown in Figure 1, the product of incorporation of lysine-C^{14} into tungstic acid-insoluble material by the cell-free *E. coli* system, with poly A as messenger, yielded radioactive di- and trilysine upon digestion with trypsin: little or no radioactive lysine was released. The di- and trilysine peaks accounted for the bulk of the radioactivity of the tryptic digest. Prior to digestion all the radioactivity remained at the origin of the chromatogram. Since, as reported by other investigators,[7] di- and trilysine are the main products of tryptic digestion of chemically synthesized poly-L-lysine, the results provide conclusive proof that poly A directs the synthesis of poly-L-lysine by the *E. coli* system.

Experiments with poly AU: As previously reported,[5] poly AU (5:1) promotes the incorporation of asparagine, isoleucine, leucine, lysine, and tyrosine into tungstic-acid insoluble products. The polymer should also stimulate the incorporation of phenylalanine but, due to the low frequency of UUU triplets, this was not de-

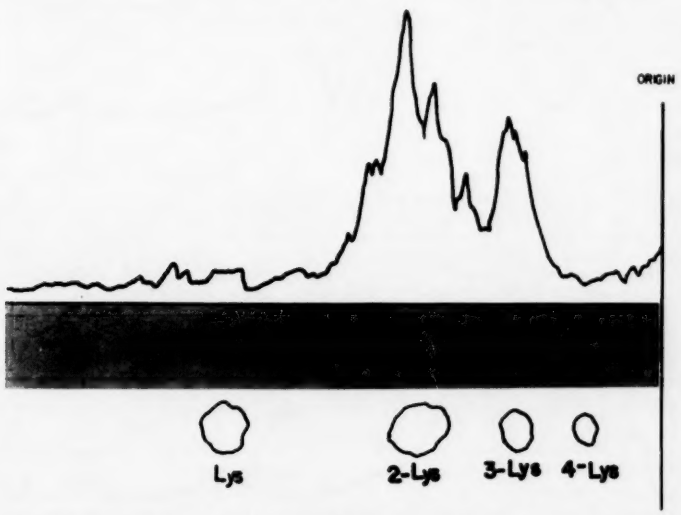

FIG. 1.—Identification of the product of lysine incorporation in the presence of poly A as poly-L-lysine. Paper chromatographic separation of products of tryptic digestion as described under *Methods*. Upper panel, actigraph tracing; middle panel, autoradiogram; lower panel, from left to right, lysine, dilysine, trilysine, and tetralysine markers.

tected. Of the above amino acids, those incorporated to a greater extent, namely, asparagine, isoleucine, and lysine, were selected for the present experiments.

Paper electrophoresis of a tryptic digest of the tungstic acid-insoluble products from an incubation with lysine-C^{14} and cold asparagine, isoleucine, leucine, phenylalanine, and tyrosine, in the presence of poly AU (5:1), yielded six major radioactive peaks, 1 to 6 from anode to cathode. Peaks 5 and 6 were in the position of dilysine and trilysine, respectively. Under the same conditions but with isoleucine-C^{14} as the only labeled amino acid (Fig. 2, upper tracing), peaks 1 through 4 were in the same positions as in the lysine-C^{14} experiment, but the radioactive peaks 5 and 6 were missing. Accordingly, in an experiment with both isoleucine-C^{14} and lysine-C^{14} (cold asparagine, leucine, phenylalanine, and tyrosine), six major radioactive peaks were obtained (Fig. 2, lower tracing). Peaks 5 and 6 were again in the position of dilysine and trilysine; peaks 1 through 4 were in the position of the corresponding peaks in the isoleucine-C^{14} experiment. The actigraph tracing from an experiment with asparagine-C^{14}, lysine-C^{14}, and cold isoleucine, leucine, phenylalanine, and tyrosine, is shown in Figure 3. Six major radioactive peaks

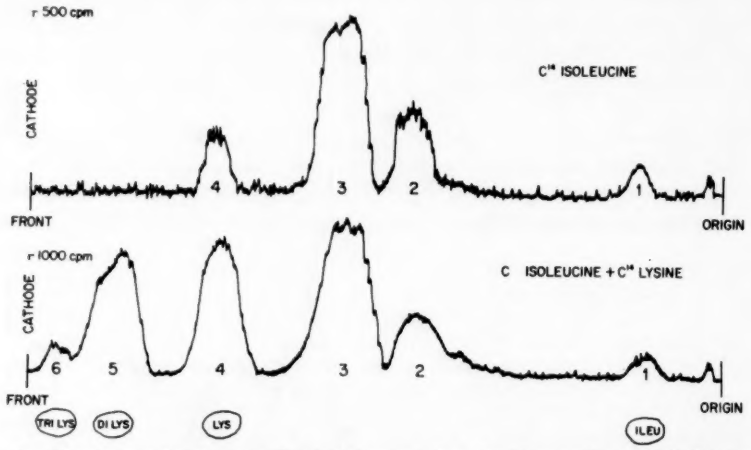

FIG. 2.—Tryptic peptides from products of incorporation of various amino acids in the presence of poly AU (5:1). Actigraph tracings of peptides separated by high voltage paper electrophoresis as described under *Methods*. Upper tracing, the incubation mixture contained isoleucine-C^{14} and cold asparagine, leucine, lysine, phenylalanine, and tyrosine. Lower tracing, the incubation mixture contained isoleucine-C^{14}, lysine-C^{14}, and cold asparagine, leucine, phenylalanine, and tyrosine. Bottom panel, from left to right, trilysine, dilysine, lysine, and isoleucine markers.

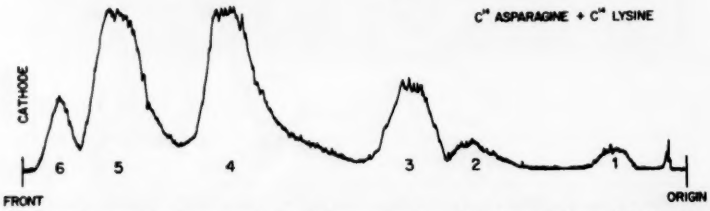

FIG. 3.—Tryptic peptides from products of incorporation of various amino acids in the presence of poly AU (5:1). The incubation mixture contained asparagine-C^{14}, lysine-C^{14}, and cold isoleucine, leucine, phenylalanine, and tyrosine. Other details as in Fig. 2.

were again obtained, of which peaks 5 and 6 corresponded to dilysine and trilysine. Prior to digestion with trypsin the radioactive material remained in all cases at the origin of the electropherogram. It should be pointed out that peaks 1 through 4 in all of the above experiments are probably mixtures of several peptides. The separation of individual peptides is now in progress.

In the experiments with radioactive (*a*) isoleucine + lysine and (*b*) asparagine + lysine, in which these amino acids were used at the same specific radioactivity, peaks 1 through 4 were separately eluted and the eluates worked up as described in the section on *Methods*. The eluates were hydrolyzed with hydrochloric acid, the hydrolysates subjected to paper chromatography, and the chromatograms scanned with an actigraph. All peaks yielded radioactive isoleucine and lysine in (*a*), and asparagine and lysine in (*b*). This is shown in Figure 4 for peaks 1 and 3 from each experiment. It may be seen that the proportion of lysine, relative to that of isoleucine or asparagine, was higher in peak 3 than in peak 1. It is also apparent that peak 1, which migrated at the same rate as an isoleucine marker (cf. Fig. 2), was in fact a peptide peak. Paper chromatography prior to acid hydrolysis of peak 4, which migrated on electrophoresis at the same rate as lysine, showed the presence of free lysine in this peak. It therefore consisted of a mixture of lysine and lysine-rich peptides. Since no lysine was present in Fraction B before digestion with trypsin, the amino acid must have been released during tryptic hydrolysis of the sample.

Further work aimed at closer identification of the tryptic peptides from incubations with poly AU (5:1), in the simultaneous presence of C^{14}-labeled asparagine, isoleucine, leucine, lysine, phenylalanine, and tyrosine, is under way. In the meantime the above experiments leave little doubt that the adenylic acid-rich copolymer poly AU (5:1) directs the synthesis of lysine-rich copolypeptides. This follows

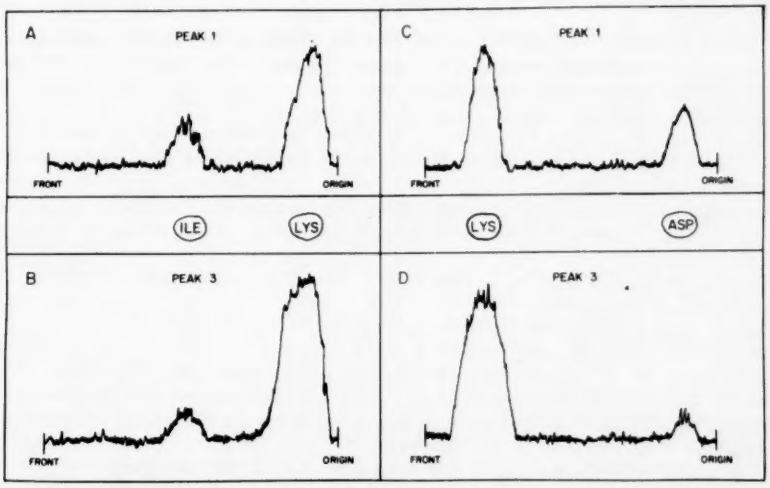

FIG. 4.—Amino acids released by acid hydrolysis of tryptic peptides from products of incorporation of various amino acids in the presence of poly AU (5:1). Actigraph tracings of paper chromatograms as described under *Methods*. *A* and *B*, peaks 1 and 3 from experiment with isoleucine-C^{14} and lysine-C^{14}. *C* and *D*, peaks 1 and 3 from experiment with asparagine-C^{14} and lysine-C^{14}. The position of amino acid markers is shown on the middle strip.

from the fact that the reaction products are cleaved by trypsin to yield, besides di- and trilysine, peptides containing isoleucine and lysine, and asparagine and lysine. These observations provide experimental support for one of the basic premises underlying the use of synthetic copolynucleotides in studies of the amino acid code.

Discussion.—End-group assays were performed by the dinitrofluorobenzene procedure in several experiments with poly A. The average chain length of the polylysine was from 14 to 17 residues. Since the average chain length of the poly A ($S_{20,w} = 7.4$ *S*) was probably above 200, this result suggests that the poly A, the polylysine, or both are cleaved by nucleases and/or peptidases in the crude *E. coli* fractions. Cleavage of poly U, largely to uridine 5′-monophosphate, by the *E. coli* preparations has been observed by other investigators.[15, 16] On incubation of C^{14}-labeled poly A with the *E. coli* system, under the conditions of polylysine synthesis, we have found that 40–60 per cent of the radioactivity becomes acid-soluble, mainly as adenosine 5′-monophosphate, within a few minutes. Further work is required for an adequate correlation of the length of the synthesized polypeptides with that of their polynucleotide templates.

Summary.—Tryptic hydrolysis of the tungstic acid-insoluble product of lysine incorporation by the cell-free *E. coli* system, in the presence of poly A, yielded dilysine and trilysine, thus characterizing the product as poly-L-lysine. Tryptic hydrolysis of the tungstic acid-insoluble products synthesized, in the presence of poly AU (5:1), from a mixture of asparagine, isoleucine, leucine, lysine, phenylalanine, and tyrosine, some of which were labeled with C^{14}, yielded a series of radioactive peptides which, upon acid hydrolysis, released radioactive lysine and isoleucine, or lysine and asparagine. This shows that poly AU, a copolynucleotide of adenylic and uridylic acid, directs the synthesis of lysine-containing copolypeptides by the *E. coli* system.

* Aided by a grant (A-1845) from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service. The abbreviations used, other than DPNH, which stands for reduced diphosphopyridine nucleotide, are as in previous papers (see reference 11). Preliminary report, Kaziro, Y., and A. Grossman, *Fed. Proc.*, **22**, 644 (1963).

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*DIFFERENCES IN THE AMINO ACID COMPOSITION OF TWO PURIFIED ANTIBODIES FROM THE SAME RABBIT**

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In previous studies[1,2] no significant differences could be demonstrated in the amino acid content of rabbit antibodies of different specificity. These data have been used to support the theories of antibody formation[3, 4] which postulate that specificity results from a three-dimensional rearrangement of peptide chains in γ globulin. However, a significant change in the steric arrangement of the active site might be caused by a relatively small percentage change in the total number of amino acids. Such a change could have been obscured by the experimental error in the analytical procedures used. For example, with the exception of methionine, histidine, and tryptophan, more than 40 moles of each amino acid have been recovered per mole of antibody. A change of two residues, one at each active site, would result therefore in a maximum difference of 5 per cent in the total content of these amino acids.

Recently, the experimental error in such measurements has been considerably reduced by the development of (*a*) the automatic amino acid analyzer[5] and (*b*) improved methods for the isolation of antihapten antibodies[6, 7] whose purity can be assayed by as stringent criteria as equilibrium dialysis.[8, 9] The purpose of the present research was to re-examine the amino acid composition of two rabbit antibodies with the improved analytic accuracy of these methods. Antibodies of completely unrelated specificity, one directed against the negatively charged *p*-azobenzenearsonic acid group and the other against the positively charged *p*-azophenyltrimethylammonium ion, were chosen to provide the optimum material for the detection of differences in the amino acid content. Furthermore, to minimize the possibility that the results might reflect genetic differences in the pools from which the antibodies were obtained, experiments were performed in which the two antibodies were isolated from the serum of single rabbits.

Preparation of antisera: The two immunizing antigens were synthesized by coupling (1) 10 micromoles of bovine γ globulin to 1.2 millimoles of diazotized arsanilic acid, and (2) 10 micromoles of bovine serum albumin to 0.4 millimole of the diazonium salt of *p*-aminophenyltrimethylammonium chloride. After exhaustive dialysis to remove excess reagent, the azoproteins were precipitated with alum by the method of Karush and Marks.[6] Antisera were prepared in New Zealand white rabbits which were injected with increasing amounts of antigen over a period of four weeks and exsanguinated five days after the last inoculation. Each animal received a total of 80 mg, composed either of a single antigen or of equal amounts of both antigens.

Purification of antibody: The method employed for the isolation of arsonic antibody[10] has been described in detail elsewhere.[7] Briefly, it consists of the preliminary removal of antiprotein carrier antibody and complement by the addition of 14 μg of bovine γ globulin N/ml of antisera. The antihapten antibody was then precipitated with 12–16 μg N/ml of an azoantigen in which human

fibrinogen was substituted as the protein carrier. The washed immune precipitate was dissolved in 0.025 *M* *p*-nitrobenzenearsonic acid, and the resulting solution was applied to a DEAE-cellulose column equilibrated with 0.02 *M* phosphate buffer, pH 7.2. The antibody appeared in the eluate as a single peak at column volume.

A similar procedure was used to purify the anti-*p*-azophenyltrimethylammonium antibody. After the antiprotein-carrier antibody was removed by the addition of 20 μg of bovine serum albumin N/ml, the specific antibody was precipitated with azofibrinogen at concentrations ranging from 10 to 12 μg N/ml. The immune complex was dissolved in an excess of *p*-nitro derivative of the hapten and the mixture was chromatographed on a DEAE-cellulose column under the conditions described above. Two modifications were found necessary. First, the fibrinogen was pretreated with iodoacetic acid under conditions in which only the lysyl residues were modified. The increase in net negative charge achieved by carboxymethylation permitted the fibrinogen to be retained on DEAE-cellulose even after extensive coupling to the positively charged hapten. Second, an additional passage through carboxymethyl cellulose was employed to separate the mixture of antibody and positively charged hapten obtained from the DEAE-cellulose chromatography. When the cation exchange resin was equilibrated with 0.02 *M* phosphate buffer, pH 7.2, the antibody appeared as a single peak at column volume.

Purity of Antibody Preparations.—Since the purity of the antibody preparations was critical to the evaluation of the results, it was assayed by three different methods. First, the binding capacity was measured by the equilibrium dialysis technique, using procedures that have been described previously.[7] The arsonic antibody was dialyzed against acetylated arsanilic acid in which the carboxyl carbon was radioactive. The ammonium antibody was dialyzed against *p*-nitrophenyltrimethylammonium chloride synthesized with C^{14} methyl sulfate by a modification of the method of Zaki and Fahim.[11, 12] The binding capacity was calculated from the amounts of radioactivity found on each side of the membrane at equilibrium and from the antibody concentration determined by micro-Kjeldahl nitrogen analysis. In the six preparations tested, more than 1.95 of the theoretical maximum of two hapten molecules were bound per molecule of antibody. These values indicated that the preparations were pure within the limits of detection of the method.

Second, the molecular weights of both antibodies were determined by the equilibrium method of Yphantis.[13, 14] Two preparations isolated from a single rabbit were diluted to contain 1.0, 0.5, and 0.1 mg/ml, and equilibrated with 0.1 *M* phosphate buffer, pH 7.0, and 0.15 *M* KCl. The samples and buffer blanks were centrifuged at 20,410 rpm for 24 hr at 21.5°. The data from the 1 mg/ml solutions are shown in Figure 1, where the logarithm of the change in antibody concentration as measured by fringe height is plotted as a function of r, the distance in the cell from the center of rotation. In this method the linearity of the plots is an index of the purity of the material, and the excellent linear relationship shown in Figure 1 is consistent with a highly pure preparation.

Finally, the possibility that the samples contained small amounts of impurities introduced during the purification procedures was tested by the use of radioactive reagents. The antigens employed, bovine γ globulin, bovine serum albumin, and the azofibrinogens, were trace-labeled with two to four I^{131} atoms per molecule by a modification of the method of McFarlane.[15, 16] Specific haptens containing C^{14} were synthesized as described above for the equilibrium dialysis measurements. These radioactive components were then used at the appropriate stage in the purification procedure and the amount of radioactivity in the final product was

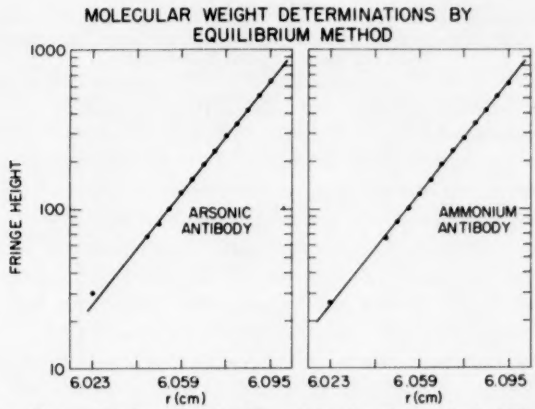

FIG. 1.—Determination of molecular weights by the method of Yphantis.[13] Since the fringe height at any point is directly proportional to the protein concentration, the measurement of fringe height at regular intervals in the cell gives the equilibrium distribution of material. The data shown in the left-hand plot were obtained with purified arsonic antibody at a concentration of 1 mg/ml. The data in the right-hand plot were obtained with purified ammonium antibody at the same concentration.

assayed. The results given in Table 1 show that the quantities of the respective protein antigens in the final products were negligible, less than 0.5% on a molar basis. The only significant contaminant was the five moles of *p*-nitrophenyltrimethylammonium chloride bound per mole of purified ammonium antibody. However, since at least 15 moles of each amino acid were recovered per mole of antibody, this amount of hapten contaminant represented only a 0.3% impurity in terms of the amino acid composition.

TABLE 1

EXTENT OF CONTAMINATION BY REAGENTS USED IN THE PURIFICATION PROCEDURE

Reagent	Ratio of Contaminant to Purified Antibody (moles/mole)	
	Arsonic antibody	Ammonium antibody
Protein carrier	3.0×10^{-4}	$<3.0 \times 10^{-7}$
Specific antigen	7.6×10^{-5}	6.3×10^{-5}
Specific hapten	$<1.0 \times 10^{-2}$	5.1×10^{-2}

Amino acid analyses: Amino acid analyses have been carried out on at least 15 different preparations of each antibody isolated from the pooled sera of immunized rabbits. The average recoveries obtained for each antibody after 20 hr of acid hydrolysis and the calculated standard errors of an individual determination are given in Table 2. The data have been expressed as moles of residue per mole of antibody and normalized to a leucine content of 89 and 91 residues for the arsonic and ammonium antibody, respectively. The values of 89 and 91 represent the average recoveries of leucine per mole of antibody as calculated from nitrogen analyses of the antibody hydrolysates and the assumptions of a nitrogen content of 16% and a molecular weight of 160,000.

To determine the total content of those amino acids known to be partially labile or resistant to 20 hr acid hydrolysis, samples of each antibody were hydrolyzed for periods of 20–96 hr. The threonyl and seryl residues in ammonium antibody were

TABLE 2

AVERAGE AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS* OF TWO PURIFIED ANTIBODIES ISOLATED FROM POOLED RABBIT SERA

Amino acid	Residues/160,000 g		Standard Error of a Single Determination	
	Arsonic antibody†	Ammonium antibody†	Arsonic antibody	Ammonium antibody
Lysine	71.4	68.7	1.66	2.13
Histidine	16.4	16.4	0.35	0.53
Arginine	45.3	41.9	1.19	1.36
Aspartic acid	105	111	0.72	0.73
Threonine	165	164	1.40	1.96
Serine	153	153	1.36	1.26
Glutamic acid	123	126	1.07	2.43
Proline	116	114	2.57	2.76
Glycine	110	111	1.41	1.08
Alanine	79.2	82.0	1.02	0.93
Half-cystine‡	43.7	44.1	0.50	0.72
Valine	127	129	2.93	2.61
Methionine	14.0	13.4	0.40	0.35
Isoleucine§	48.7	46.1	0.67	0.77
Leucine	89	91		
Tyrosine	55.9	56.5	1.54	0.83
Phenylalanine	44.2	45.4	0.56	0.41

* Hydrolysis time was 20 hr.
† Average values from the analyses of 13 different preparations.
‡ Determined as carboxymethylcysteine.
§ Sum of isoleucine and alloisoleucine.

found to be destroyed at the usual first-order rate, while those in arsonic antibody exhibited different labilities to acid hydrolysis.[16] However, extrapolation of the curves for each antibody to zero hydrolysis time gave identical values, 174 threonines and 172 serines. In addition to the losses in threonine and serine, the tyrosine recoveries were found to decrease slowly on longer hydrolysis, but even after extrapolation to zero hydrolysis time the average yield was 95% of that obtained from basic hydrolysates. No significant increases were observed in the valine and isoleucine recoveries upon prolonged hydrolysis, although Smith *et al.*[1] reported the incomplete release of these residues in 20-hr hydrolysates of rabbit antipneumococcal antibodies.

When the amino acid compositions of arsonic and ammonium antibody as given in Table 2 were corrected for the serine and threonine losses, and the tyrosine and tryptophan values from the basic hydrolysates[16] were substituted, the total residues recovered for each antibody were identical within experimental error (Table 3). Furthermore, the nitrogen content calculated from the total residues was found to be 16.4% for both antibodies. If these values were corrected for the 3–4% nitrogen present in the carbohydrate fraction, they were in excellent agreement with the assumed content of 16%, on which the determinations of the absolute leucine recoveries and the subsequent normalization procedure were based.

TABLE 3

PROPERTIES OF PURIFIED RABBIT ANTIBODIES

	Arsonic antibody	Ammonium antibody
Total residues recovered	1,458	1,465
% N in total residues*	16.4	16.4
Molecular weight at 1.0 mg/ml	163,500	163,500
Molecular weight at 0.5 mg/ml	163,500	164,500
Molecular weight at 0.1 mg/ml	159,600	160,300
Average molecular weight	162,200	162,800

* The calculation does not include the nitrogen in the carbohydrate fraction.

Further support for the amino acid compositions given in Table 2 was provided by the independent measurements of molecular weights. In Table 3 are listed the values calculated from the slopes of fringe height versus r, the experimentally determined density of the phosphate buffer solvent, and a partial specific volume for human γ globulin of 0.739.[17] The average of 162,200 for arsonic antibody and 162,800 for ammonium antibody not only demonstrated that the molecular weights of the two antibodies were identical within experimental error, but also justified the calculation of the amino acid compositions on the assumed value of 160,000.

Two features of the amino acid data were striking. The first was the over-all similarity in the compositions of the two antibodies. The second was the appearance of small, but significant differences. The arsonic antibody had a higher arginine and isoleucine content, while the ammonium antibody had a higher aspartic acid and leucine content. The significance of the difference in the arginine yields is not apparent from the standard errors shown in Table 2. However, the values given included not only the error of the analyses but also an additional pipetting error since the resolution of arginine required a separate application of the hydrolysate to the short column of the automatic analyzer. When a standard was used which was resolved on both the long and short columns or when the same pipette was employed for both applications, the error in a single arginine determination was reduced to less than 0.6 mole.

The observed differences, especially in the aspartic acid and arginine yields, were too large to be explained by the amounts of impurities found in the preparations. The possibility remained, however, that the results reflected genetic differences in the pooled sera from which the antibodies were obtained. This possibility was minimized by the data shown in Table 4, which represent the average amino acid recoveries from nine pairs of antibodies isolated from the sera of single rabbits. These analyses were performed under carefully controlled conditions; an assay of an amino acid standard was alternated with each assay of an antibody hydrolysate, and the errors in the recoveries on the short column were minimized

TABLE 4

AVERAGE AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS* OF TWO PURIFIED ANTIBODIES ISOLATED FROM THE SAME RABBIT

Amino acid	Residues/160,000 g		Standard Error of a Single Determination	
	Arsonic antibody†	Ammonium antibody†	Arsonic antibody	Ammonium antibody
Lysine	69.6	69.4	0.87	0.56
Histidine	16.4	16.6	0.23	0.16
Arginine	44.7	42.5	0.46	0.49
Aspartic acid	106	110	0.75	1.07
Threonine	162	162	3.05	2.53
Serine	151	151	1.90	1.70
Glutamic acid	125	127	2.15	2.00
Proline	109	110	2.74	2.35
Glycine	110	110	1.73	1.50
Alanine	81.1	81.4	0.86	1.65
Valine	128	128	1.80	1.70
Methionine	13.8	13.5	0.36	0.53
Isoleucine‡	48.4	46.4	0.93	0.96
Leucine	89	91		
Tyrosine	56.1	56.2	1.07	0.61
Phenylalanine	44.3	44.9	0.49	0.43

* Hydrolysis time was 20 hr.
† Average values from the analyses of nine different preparations.
‡ Sum of isoleucine and alloisoleucine.

by use of a single pipette for the application of the sample. It may be seen that the amino acid compositions of the two antibody preparations from individual rabbits were identical to those determined with pooled samples. Thus, the higher content of arginine and isoleucine in the arsonic antibody and the higher content of aspartic acid and leucine in the ammonium antibody were confirmed. Furthermore, using the standard statistical calculations, the differences in the arginine and aspartic acid values were shown to have a 99.9% probability of being outside experimental error.

Discussion.—The data presented above demonstrate for the first time significant differences in the amino acid composition of two purified rabbit antibodies. These results can be interpreted in two general ways. The first possibility is that the differences are related to antibody specificity and represent a change either in the contact amino acids at the active site or in residues which indirectly determine the steric arrangement of the active site. This interpretation would, of course, require that antibody synthesis be under genetic control and not result merely from a changed arrangement of the same peptide chains of γ globulin. The modification of the properties of a protein by a change in relatively few residues has already been demonstrated in the well-known studies of hemoglobin produced in sickle-cell anemia.[18, 19]

The second possibility is that the amino acid differences are not related to antibody specificity, but represent pools of different γ globulin molecules. The existence of γ globulin allotypes in rabbits has already been established by studies of their antigenic properties.[20–22] It is possible therefore that any one antibody activity is segregated or partially segregated in one type of globulin and that the amino acid differences merely reflect such a segregation. Since the same differences in composition were observed whether the antibodies were prepared from pooled sera or from the serum of a single animal, this interpretation requires that the two types of globulin are produced in all the animals used.

Support for the first hypothesis is provided by a correlation of the observed amino acid differences with the available evidence concerning the chemistry of the active sites. The higher aspartic acid content of the ammonium antibody is consistent with previous findings that its active center contains a negatively charged group. Grossberg and Pressman[23] showed that treatment of ammonium antibody with diazoacetamide destroyed the binding capacity and that the loss was prevented when the reaction was carried out in the presence of the homologous hapten. Analyses of the treated antibody indicated that the major change was a reduction in the number of free carboxyl groups. Similarly, the higher arginine content observed for arsonic antibody is consistent with the present chemical evidence for the structure of its active site. The pH dependence of the binding capacity indicated the participation of side chains with a pK of 10 or above. The presence of tyrosine, first suggested by Pressman and Sternberger,[24] was established by iodination and photooxidation studies in our laboratory.[25] The possible contribution of a lysyl residue was eliminated by Wofsy and Singer[26] who demonstrated that exhaustive amidination of the ϵ amino groups reduced the binding capacity by only 27%. These results were confirmed in our laboratory by the use of iodoacetic acid under conditions in which only lysyl residues were modified. It was found that 17% of the binding capacity was retained after carboxymethylation of all the ϵ amino

groups.[27] Since a single reactive tyrosine would not provide the degree of specificity observed for the arsonic antibody-antigen interaction,[7] the presence at the active site of the remaining positively charged determinant group, arginine, appears highly probable.

Further support for the first hypothesis is provided by genetic analyses of arsonic and ammonium antibodies isolated from the same rabbit. The results showed that the allotypic specificity of the two preparations was identical in every case although a variety of single and doubly heterozygous animals was represented. Furthermore, the allotypy of the purified antibodies corresponded with that of the whole serum except in two animals in which there was a deletion in both antibodies.[28]

The alternative hypothesis that the amino acid compositions reflect pools of different γ globulin molecules finds support in the considerable data indicating the microheterogeneity of both normal and immune γ globulin. In particular, the variation in the net charge among rabbit globulin molecules has been well established by use of zone electrophoresis and elution from carboxymethyl cellulose.[29, 30] These investigations have been extended to the univalent fragments liberated by papain, since they have been shown to differ in mobility in the same manner as the undigested globulin. Mandy and Nisonoff[31] have recently reported small differences in the charged amino acid content of the fragments which are in the direction expected from the mobilities. These data are consistent with the hypothesis that differences, at least in the charged amino acid recoveries, are not related to antibody specificity. However, Feinstein[30] found that the electrophoretic heterogeneity of γ globulin and its fragments could be accounted for by differences observed in the amide content rather than in the number of charged amino acid residues.

It is apparent from the above discussion that, although the finding of differences in the amino acid composition is suggestive, a definitive mechanism is not yet available. Amino acid analyses of several other antibodies, particularly one directed against an uncharged moiety, should help to distinguish between the alternative hypotheses. These experiments are in progress.

Summary.—Small but significant differences have been found in the amino acid composition of two antibodies purified from pooled sera or from the serum of the same rabbit. The antibody directed against the negatively charged *p*-azobenzenearsonic acid group had a higher arginine and isoleucine content, while the antibody directed against the positively charged *p*-azophenyltrimethylammonium ion had a higher aspartic acid and leucine content. The similarity in the recoveries of the remaining amino acids was striking, and the molecular weights of the two antibodies were shown to be identical within the limits of the method. The possible relationship of these differences in amino acid composition to antibody specificity and to the microheterogeneity of γ globulin is discussed.

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*PHYSICAL AND CHEMICAL PROPERTIES OF RNA FROM THE BACTERIAL VIRUS R17**

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In a previous communication[1] we have described R17, a newly isolated bacterial virus which contains RNA. The present report concerns properties of the purified viral RNA. It will be shown that R17 RNA can be obtained as a homogeneous, high molecular weight particle whose configuration in solution is a function of ionic strength and temperature.

Methods.—Purification of the virus: R17 virus was grown on *E. coli* K-12 (Hfr, methionine$^-$). It was purified by acid precipitation (of impurities), ammonium sulfate fractionation, and differential centrifugation as has already been described.[1] The purified virus was checked for infectivity by the agar layer procedure of Adams[2] and for homogeneity in the analytical ultracentrifuge with both schlieren and ultraviolet optics.

Isolation of R17 RNA: RNA was isolated from the virus by a phenol procedure

based on those of Gierer and Schramm,[3] Fraenkel-Conrat *et al.*,[4] and Sreenvasaya and Pirie.[5] A solution of the virus in appropriate buffer, mixed with an equal weight of fractionated bentonite and then made up to 0.2 per cent with respect to sodium dodecyl sulfate, was chilled in ice and then shaken vigorously for about 5 min with an equal volume of redistilled phenol saturated with water. The mixture was separated by centrifugation. The aqueous layer was removed, and the extraction with phenol was repeated twice. The aqueous layer was extracted thrice with ether. Residual ether in the aqueous RNA solution was removed by bubbling nitrogen through it. The solution was stored at −15°C. Thereafter, in some cases, the RNA was precipitated at 5°C with two volumes of absolute ethanol, spun down, dissolved in the desired buffer, dialyzed against the same buffer 12–24 hr at 5°C, spun at 20,000 rpm for 20 min in the No. 30 rotor of a Spinco Model L centrifuge to remove any residual finely divided bentonite, and then stored at −15°C.

Detection of contaminants: Samples of R17 RNA were examined for DNA content with the diphenyl amine reaction;[6] a standard curve was derived with calf thymus DNA. Protein content was measured with the Lowry reagent;[7] bovine serum albumin was used as a standard.

Absorption spectra: Ultraviolet and visible spectra were determined in a Cary Model 11 spectrophotometer equipped with quartz absorption cells of 1 cm light path. The kinetics of hydrolysis by ribonuclease were followed with the same instrument.

Base composition: Base composition was determined by the method of Markham and Smith.[8] Purine bases and pyrimidine nucleotides were identified by their absorption spectra. Their abundance was determined from their absorbancy indices.[9]

Measurement of concentration: RNA concentration was routinely determined by ultraviolet absorbance measurement. The absorbancy index applied for the RNA was determined from the weighted absorbancy index of the mononucleotide residues (weighted according to the base composition) and from the hyperchromicities observed after complete alkaline hydrolysis of the RNA.

The buffer used most frequently was 0.1 *M* KCl + 0.01 *M* K phosphates, pH 7.0 at 20°C. Hereafter, it is called KCl-phosphate buffer.

Sedimentation analyses: Sedimentation behavior of the RNA was followed with ultraviolet and schlieren optics in the Spinco Model E analytical ultracentrifuge with 12 or 30 mm, 4° sector cells at speeds of 50,740 or 59,780 rpm, and at a temperature of 20.0 ± 0.01°C. Ultraviolet pictures were traced densitometrically with a Jarrell-Ash microdensitometer. Sedimentation velocity measurements made at concentrations less than 0.003% and appropriately corrected for solvent viscosity are designated $s^{\circ}_{20,w}$.

Viscosity studies: Flow rates were measured to 0.1 sec in a modified Ostwald viscometer immersed in an oil bath held at 20° ± 0.01°C (flow time of about 315 sec for water).

Absorbance-temperature experiments: Measurements of ultraviolet absorbance as a function of temperature were made in a Beckman DB spectrophotometer. The temperature of the cell chamber was controlled by circulating water from a bath regulated to 0.1°C. The temperature of the RNA solution in the cell was read to 0.2°C with a dipping thermistor.

Results.—Ultraviolet spectrum: The ultraviolet absorption spectrum of R17 RNA is typical of that of high molecular weight RNA. It exhibits a maximum at 258 mμ and a minimum at 230 mμ. The ratio of the absorbancy at 260 mμ to that at 230 mμ, in KCl-phosphate buffer at pH 7.0, is 2.28; the 260/280 ratio is 2.23.

Base composition: The base composition, determined in duplicate, is tabulated in Table 1. It should be noted that the value for cytidylic acid may be slightly low because of deamination under the conditions of hydrolysis.

Absorbancy index of R17 RNA: The absorbancy at 260 mμ of an aliquot of R17 RNA in KCl-phosphate buffer was compared with the absorbancy of an identical quantity of R17 RNA in 0.3 *N* NaOH, 20°C, which had been heated for 24 hr at 37°C to convert the RNA to mononucleotide form. The absorbancy of the second aliquot was 1.35 times as great as that of the first. It is known that 1 mg/ml of nucleotide residues, present in the same proportion as they exist in R17 RNA, has an absorbancy of 31.5 (calculated from Table 3, page 513, reference 9). The absorbancy index of R17 RNA in KCl-phosphate buffer is thus 31.5/1.35 = 23.3 cm^2/mg.

Effect of ribonuclease: Pancreatic ribonuclease (0.35 μg/ml) degrades R17 RNA (35 μg/ml) in a complex kinetic way as followed by increase of ultraviolet absorbancy. The absorbancy reaches a maximum about 1.3 times its initial value within 60 min and remains unchanged thereafter.

Contamination of the preparation with protein and DNA: Neither protein (Lowry reagent) nor DNA (diphenylamine reagent) could be detected in aliquots of the RNA preparations. The maximum DNA content could be 0.6 per cent of that of RNA and the maximum protein content 0.5 per cent of that of RNA.

Sedimentation: The homogeneity of the RNA in different buffers was studied in sedimentation velocity runs. The RNA was essentially homogeneous as is evident from the single sharp boundary found both with schlieren (Fig. 1) and ultraviolet (Fig. 2) optics. Frequently there is also a slowly sedimenting diffuse boundary indicative of heterogeneous material 5–10% as abundant as the major sedimenting component. This material is more evident with schlieren optics (see Fig. 1), probably because of the Johnston-Ogston effect. Because its proportion varies among batches, it is presumed to be an RNA degradation product brought about by nuclease action during or prior to the extraction of the RNA. The patterns of samples heated to 60°C for 10 min and then chilled quickly exhibit very slightly more of the slower moving material. Occasionally, preparations exhibit two distinct boundaries in addition to the main boundary.

As is typical of ribonucleic acids the sedimentation rate of R17 RNA varies with ionic strength (Table 2), reflecting the dependence of configuration on the charge distribution in the vicinity of the RNA. The highest sedimentation rate, $s^\circ_{20,w}$ = 26.2 S, was measured in KCl-phosphate buffer.

TABLE 1

BASE COMPOSITION OF R17 RNA

Experiment	A	G	C	U
I	0.237	0.257	0.247	0.259
II	0.225	0.270	0.251	0.255
Average	0.231	0.263	0.249	0.257

The base composition was determined in duplicate on the acid hydrolysate of the RNA. The results agree with the values of Paranchych and Graham[24] who analyzed an alkaline hydrolysate. Their values for A:G:C:U are 0.226:0.271:0.248:0.255.

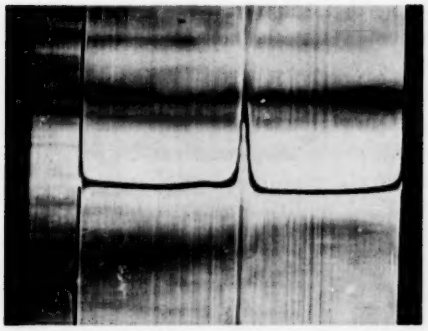

FIG. 1.—Schlieren sedimentation diagram of R17 RNA (approximately 1.2 mg/ml) in KCl-phosphate buffer in the 30 mm cell taken 19 min after reaching top speed of 50,750 rpm.

FIG. 2.—Densitometric tracing of an ultraviolet ultracentrifuge pattern of R17 RNA (approximately 48 μg/ml) in 0.01 *M* K phosphate, 11 min after reaching top speed of 59,780 rpm in a 12 mm cell. The two regions of low opacity (on the left and on the right) correspond to the reference holes of the centrifuge cell. The sharp spike on the left side of the diagram corresponds to the meniscus. The region of opacity increase in the left center of the diagram corresponds to the boundary of the RNA. The region of opacity increase on the right side of the diagram corresponds to the cell bottom.

Viscosity: Intrinsic viscosity measurements were made under conditions corresponding to those for sedimentation as shown in Table 2.

Molecular weight of R17 RNA can be estimated from the intrinsic viscosity $[\eta]$ and the sedimentation coefficient, $s^\circ_{20,w}$, by means of the Scheraga-Mandelkern equation[10]

$$M^{2/3} = \frac{s^0_{20,w}\,[\eta]^{1/3}\,\eta o\,N}{\beta\,(1 - \bar{v}\rho)}$$

Here, ηo, ρ, and N are solvent viscosity and density and Avogadro's number, respectively. The partial specific volume, $\bar{v}$, of the RNA was taken to be 0.53 cc/g (the average of values quoted for RNA by Tissières,[11] Kurland,[12] and Bruening[13]). The appropriate value for Scheraga and Mandelkern's parameter, β, is not known. Its minimum value from theory is 2.12×10^6 for particles which behave hydrodynamically as spheres. Its value is unlikely to be greater than 2.50×10^6. Taking these values as extremes, the molecular weight of R17 RNA as determined by sedimentation and viscosity is in the range 0.95–1.21 $\times 10^6$ in low ionic strength buffer, and 1.08–1.37 $\times 10^6$ in high ionic strength buffer. Enger *et al.*[1] determined that R17 virus contains about 1.3×10^6 atomic mass units of RNA. Thus, it is likely that R17 RNA exists as a single piece.

Intrinsic viscosity also furnishes an approximate measure of radius of gyration from the Flory-Fox equation.[14]

$$R_G = \frac{M^{1/3}\,[\eta]^{1/3}}{6^{1/3}\,\Phi^{1/3}}$$

TABLE 2

CHARACTERISTICS OF R17 RNA IN SEVERAL MEDIA

Medium	$s^\circ_{20,w}$ (S)	$[\eta]$ (dl/g)	M	R_G (A)
0.01 *M* K phosphate, pH 7.0	18.4	1.37	0.95–1.21x10^6	350–370
0.1 *M* KCl + 0.01 *M* K phosphate, pH 7.0	26.2	0.35	1.08–1.37x10^6	220–230

The Flory-Fox parameter,[15] $\Phi^{1/3}$, is known to vary from 1.25×10^7 to 1.40×10^7, but its value under our solution conditions is not known. We have assumed 1.32×10^7. Table 2 shows values for R_G under several conditions. R_G is being determined independently from low angle X-ray scattering data.

Absorbancy-temperature experiments: Secondary structure of R17 RNA was explored mainly with absorbancy-temperature profile measurements.[16,17] Absorbancy at 260 mμ was determined as a function of temperature under several conditions of solution. Correction was made for thermal expansion of water.

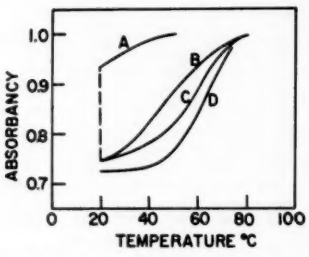

FIG. 3.—Relative-absorbancy-temperature profile of R17 RNA under various conditions. (*A*) in 6 *M* urea in KCl-phosphate buffer; (*B*) in 0.01 *M* K phosphate buffer; (*C*) in KCl-phosphate buffer; (*D*) within the intact virus itself, in KCl-phosphate buffer. Curve *D* is corrected for absorbancy of the protein.

R17 RNA in KCl-phosphate buffer has an unusually high resistance to absorbancy change with temperature. The midpoint, T_m, of the thermal transition is 58°C (Fig. 3). The absorbancy does not increase significantly below 30°C. (The absorbancy of tobacco mosaic virus RNA, under comparable conditions, starts to increase at 20°C.) The upper plateau of the sigmoid absorbancy-temperature curve was not reached. However, the maximum absorbancy is likely the same as it is in several buffers of low ionic strength and in urea (Fig. 3). There is little change in the absorbancy-temperature curve upon addition of 0.001 *M* Mg^{++}. T_m for RNA in 0.01 *M* K phosphate, pH 7.0, is lower than in KCl-phosphate buffer—its value is about 48°C. All of these transitions are reversible. However, on holding RNA solutions at high temperature (e.g., 80°C) for an hour or so, the absorbancy-temperature curves upon cooling are parallel to the heating profiles, but are shifted slightly toward higher temperature and the absorbancies are slightly lower.

The absorbancy at 20°C in 6 *M* urea + KCl-phosphate buffer is 1.25 times as great as is a comparable control solution in KCl-phosphate buffer alone. Upon heating, the absorbancy increases further, leveling off at a hyperchromicity of 1.34.

The absorbancy of R17 virus itself in KCl-phosphate buffer does not increase upon heating below 40°C. It then increases rapidly, reaching 1.33 times its original value at 73°C without evidence of leveling off (Fig. 3). Because the virus precipitates at high temperature, absorbancy was also measured in the nonabsorbing region of the spectrum at 320 mμ to detect light scattering indicative of a precipitate. Absorbancy in this region begins at 73°C.

Reaction of R17 RNA and the intact virus with formaldehyde: Formaldehyde reacts with free nucleic acid[18,19] and with nucleic acid in viruses,[20,21] increases the absorbancy, and shifts the absorption maximum toward longer wavelengths.

The absorbancy at 260 mμ of R17 RNA in KCl-phosphate buffer is increased by 5.5% upon reaction with 1% HCHO at 20°C for 24 hr, by 16% at 37°C, and by 30% at 80°C. In the latter case the absorbance maximum is shifted from 258 mμ to 262 mμ.

At low temperatures and in concentrated salt solutions, 1 per cent formaldehyde is not expected to unfold RNA, and hence the extent of the reaction between RNA

and formaldehyde under these conditions indicates the number of free amino groups (i.e., the number which are not hydrogen bonded with other groups within the RNA). We compared (by absorbancy increase at several wavelengths[19]) reaction of the RNA with formaldehyde at 20°C and at 70°C. At 70°C the RNA is almost entirely randomly coiled in the presence of 1 per cent formaldehyde and consequently almost all amino groups should be reactive. Table 3 shows that, at 20°C, in KCl-phosphate buffer about 28 per cent of the amino groups are free to react with formaldehyde. Clearly, the validity of this conclusion depends on the assumption that *extent of formaldehyde reaction* and *absorbancy change at a given wavelength* are linearly related.

TABLE 3

ESTIMATION OF FREE AMINO GROUPS

Wavelength	A	B	C	D	$\frac{A-B}{C-D} \times 100$
270	0.430	0.406	0.645	0.579	36.4
275	0.345	0.325	0.547	0.474	27.4
280	0.266	0.243	0.439	0.356	27.7
285	0.185	0.166	0.321	0.238	22.5
					Avg. 28.5%

Column A is absorbancy after reaction with formaldehyde at 20°C for 24 hr. Column B is absorbancy at 20°C prior to the reaction. Column C is absorbancy after reaction with formaldehyde at 70°C. Column D is absorbancy at 80°C prior to the reaction. The latter temperatures were chosen for the following reasons: judging from absorbancy-temperature profiles, at 70° in the presence of formaldehyde and at 80° in the absence of formaldehyde, the RNA has reached about 95% of its maximum hyperchromicity. The latter temperatures are thus close to the temperatures required for complete conversion to the random coiled configuration but not so high as to cause extensive thermal breaking of the RNA.

The intact virus reacts less with formaldehyde than does its free RNA and shows an 8 per cent increase in absorbancy at 260 mμ in 24 hr at 37°C. It may be inferred that the decreased reactivity of RNA inside the virus is due to the existence of more extensive hydrogen bonded regions.

Calculation of RNA in helical form: It is a reasonable assumption that RNA at high temperature is completely in a random coiled configuration and that at lower temperature it assumes, in part, a helical configuration. Because the helical configuration has a lower absorbancy at 260 mμ, the depression of the absorbancy below its value for a random coil may be taken as a measure of the fraction of residues which participate in the helix.[16] The average absorbancy at 260 mμ of poly (A + U) and poly (I + C) in helical form is 0.667 times the random coil value. These polymers are supposed to simulate the behavior of RNA, and thus for RNA a decrease in absorbancy to 0.667 of the random coiled absorbancy is generally accepted as evidence of complete conversion to helical form.[19] We have indicated that R17 RNA in KCl-phosphate buffer absorbs 0.745 times as much as it does in urea at high temperature (in which it doubtless is in the random coiled configuration). Thus, in the phosphate buffer at 20°C, 76 per cent of the R17 RNA bases presumably are in a helical configuration. Similarly, RNA in the intact virus in KCl-phosphate buffer absorbs 0.727 times as much as it does in random coiled configuration. Within the virus, the RNA presumably is 82 per cent in helical form.

The data on reactivity of the RNA with formaldehyde are qualitatively consistent with these conclusions. Table 3 indicates that approximately 72 per cent of the amino groups of the RNA in KCl-phosphate buffer are unreactive and thus are

presumably in a helical configuration. Similarly, the RNA within the virus is rather unreactive and thus presumably largely helical.

Discussion.—Physical studies on RNA isolated from R17 phage show that it consists primarily of homogeneous units of molecular weight of about 1.2×10^6. The correspondence of this value with the RNA content of the virus suggests that there is a single RNA molecule per virus particle. This observation conforms with the known integrity of high molecular weight viral RNA's.

Recent studies[22,23] suggest that pre-existent breaks of a small number of covalent bonds might not be evident in purified RNA because extensive hydrogen bonding and other secondary interactions would hold the pieces together. However, such RNA agglomerates would be expected to dissociate when heated above the RNA melting temperature. The fact that R17 RNA in KCl-phosphate buffer shows little slowly sedimenting material after heat treatment indicates that there are few chain breaks.

The occasional presence of discrete, additional components in slightly degraded RNA suggests that nucleases may effect a single or several scissions in the polynucleotide strand.

The base composition of the RNA determined by a procedure which involved acid hydrolysis is in good agreement with that recently reported by Paranchych and Graham[24] whose procedure involved alkaline hydrolysis. The four bases are present in almost equal amount, suggesting that the RNA could be extensively hydrogen bonded. The temperature-absorbancy profiles confirm that this is the case in concentrated salt solutions. Furthermore, measurable melting does not occur below 30°C, indicating that the RNA has relatively few weakly-held double-stranded regions. T_m of the RNA in 0.01 *M* K phosphate is about 10°C lower than it is in 0.01 *M* K phosphate + 0.1 *M* KCl. This is consistent with the 3-fold higher intrinsic viscosity and the one-third lower $s_{20,w}$ in the latter medium. All of these data signify a less compact structure in dilute salt solutions.

Summary.—RNA isolated by phenol extraction from the bacterial virus R17 has been found to consist essentially of a single homogeneous species. Its molecular weight, based on sedimentation and viscosity in several media, is in the range of $1.0–1.4 \times 10^6$. Physicochemical studies relating to RNA configuration in solution and in the intact virus have indicated that, in concentrated salt solutions and in the intact virus, the RNA is largely in an ordered, presumably helical configuration.

We are indebted to Dr. Angus Graham for giving us R17 virus and its host, and to W. M. Stanley, Jr., for discussions concerned with the isolation of RNA.

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*STUDIES ON THE FORMATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID, II. DEGRADATION OF HOST RIBONUCLEIC ACID FOLLOWING INFECTION**

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The synthesis of TMV-RNA[1] does not proceed via cell DNA. This conclusion is based on the following experimental evidence:[2] DNA, isolated from healthy and infected tobacco leaves, has similar purine and pyrimidine composition, and no differences were observed in the uptake of P^{32} into DNA of healthy and infected leaves, suggesting that no new cell DNA is formed following infection with TMV. Examination of the nucleic acid preparations obtained from infected tobacco leaves did not reveal the presence of DNA-RNA hybrids; this might have been the case if the formation of viral RNA involved DNA. Since TMV-RNA is the carrier of genetic information, its duplication might follow the sequence of information flow RNA → RNA. Even though these findings exclude the involvement of DNA in the synthesis of TMV-RNA, it might still be possible that the cell nucleus is the site of its formation.

TMV is a rod-shaped virus which is made up of 5% RNA and 95% protein.[3–6] Its formation in the cell involves the synthesis of considerable amounts of RNA and protein. Since the microsomes are the site of protein synthesis, it is of interest to study the effect of TMV infection on these particles. The results presented in this communication show that the microsomes of the cytoplasmic fraction of tobacco leaf are rapidly degraded following infection with TMV, and the breakdown products of their RNA are utilized in the synthesis of TMV-RNA.

Experimental.—TMV inoculum for infecting the plants: The common strain of TMV used in this investigation was kindly supplied by Dr. F. O. Holmes of The Rockefeller Institute. TMV inoculum used for infecting the tobacco plants was prepared as follows: 2 gm of infected Turkish

tobacco leaves, showing distinct symptoms of mosaic disease, were ground in a mortar with carborundum and 50 ml of 0.1 *M* sodium phosphate buffer at pH 7.0. The ground material was strained through two layers of cheesecloth. The inoculum was prepared a few minutes before it was needed for infecting the plants.

Tobacco plants: *Nicotiana tabacum* var. Turkish, used in these studies, were grown in a greenhouse. These were kindly provided by Dr. Armin Braun of The Rockefeller Institute.

The plants, approximately 12 weeks old, were transferred to a room in which the temperature was maintained at 25°C. They were illuminated for 14 hr a day. Plants of the same size and general appearance were selected and bottom leaves were removed and discarded. The remaining leaves were dusted with carborundum and were rubbed with the TMV inoculum, prepared as described above. Control plants were rubbed with carborundum and 0.1 *M* sodium phosphate buffer at pH 7.0. The leaves were afterward rinsed with water. Leaves of the same size (approximately 10 cm long and 8 cm wide) were harvested at intervals of time up to 28 days and the microsomal RNA and TMV-RNA were estimated as described below.

Isolation of microsomes and TMV from infected tobacco plants: Unless otherwise stated, all operations were conducted between 0°and 4°. Immediately after picking the leaves, their midribs were removed and the leaf blades were quickly cut into small pieces. 2 gm portions were placed in a homogenizer tube of 15 ml capacity fitted with a teflon pestle. The leaves were ground for 5 min with 5 ml of 0.5 *M* sucrose containing 0.01 *M* tris-HCl buffer at pH 8.0 and 0.002 *M* $MgCl_2$. The ground material was centrifuged for 20 min at 20,200 × *g* in the refrigerated Servall centrifuge. The residue was washed twice with 3 ml of the above grinding medium. The combined supernatants were centrifuged for 2 hr at 105,000 × *g* in the No. 40 rotor of the Spinco Model L ultracentrifuge. After draining the supernatant thoroughly, the pellet was suspended in 3 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA, and centrifuged for 20 min at 12,100 × *g* in the refrigerated Servall centrifuge. The residue was extracted twice with 2 ml portions of the above phosphate buffer. In the combined extracts the microsomal RNA and TMV-RNA were determined using the procedures described below.

Estimation of microsomal RNA and TMV-RNA: To 3 ml of the above extract containing microsomes and TMV was added, at 0°, 0.6 ml of 40% TCA with mixing. After 15 min, the mixture was centrifuged for 10 min at 7,900 × *g* in the refrigerated Servall centrifuge. The precipitate was washed three times with 1 ml of cold 6% TCA, and twice with absolute alcohol. It was finally washed once with a mixture of absolute alcohol and chloroform (1:1), and once with ether at room temperature. The precipitate was then air-dried, dissolved in 0.3 ml of 1.0 *N* NaOH, incubated at 30° for 24 hr, and neutralized with 2.0 *N* HCl. A 0.4 ml sample of the neutralized hydrolysate was placed on Whatman No. 3 MM filter paper as a band (10 cm long) and dried in a current of air at room temperature. The chromatogram was developed in the isopropanol-water-ammonia solvent system[7] for 24 hr, dried at room temperature, and the nucleotides were located under ultraviolet light. In this solvent system the alkali-hydrolyzed RNA gives two bands: one containing guanosine 2′(3′−)-phosphate, and the other containing a mixture of 2′(3′−)-phosphates of adenosine, cytidine, and uridine. These two bands were eluted together by running distilled water through them for 4 hr. A blank filter paper of the same dimensions, which was cut out of Whatman No. 3 MM filter paper subjected to the above chromatographic procedure, was likewise eluted. The eluates were made up to a suitable volume and their absorptions were measured in the Beckman Spectrophotometer at 260 mμ. A known amount of TMV-RNA, subjected to the above procedure, was used as a standard in calculating the RNA content of the sample.

This procedure gives the combined value for microsomal RNA and TMV-RNA of the infected leaf. The value for microsomal RNA was obtained by subtracting the value for TMV-RNA, estimated using the procedure described below.

Estimation of TMV-RNA: To 3 ml of the above extract containing microsomes and TMV were added 0.05 ml of pancreatic RNAase (400 μg, a large excess to ensure complete degradation of microsomal RNA) and a drop of chloroform to prevent microbial growth. The extract contains a sufficient amount of leaf nuclease to degrade microsomal RNA, and pancreatic RNAase was added to hasten this degradation. Under these conditions TMV is not degraded. The mixture was incubated at room temperature for 24 hr. At the end of the incubation period the tube was transferred to an ice bath, and 0.6 ml of cold 40% TCA was added to this mixture with

shaking. After 15 min, the precipitate was recovered by centrifugation. It was washed and air-dried as described above. The RNA present in the precipitate was determined by employing the alkali digestion procedure and chromatography described above.

Estimation of total RNA of tobacco leaf: Unless otherwise stated, all operations were done at 0–4°. Immediately after picking the leaves, their midribs were removed and cut into small pieces. One gm of the material was homogenized as described above with 3 ml of 6% TCA. The homogenate was centrifuged for 20 min at 7,900 × *g* in the refrigerated Servall centrifuge. The precipitate was washed three times with 2 ml of 6% TCA and three times with absolute alcohol. The washing of the precipitate with absolute alcohol was continued at room temperature until the last wash fluid was colorless. This was then washed once with a mixture of alcohol and chloroform (1:1), twice with ether, air-dried, and incubated with 1 ml of 1.0 *N* NaOH for 24 hr at 30°. At the end of the incubation period, the hydrolysate was centrifuged for 10 min in the clinical centrifuge at top speed. The supernatant was drawn out with a capillary. The residue was washed twice with 0.2 ml of 1.0 *N* NaOH. The washings were combined with the supernatant and neutralized with 2.0 *N* HCl. The rest of the procedure involving chromatography of the neutralized hydrolysate of RNA, elution of nucleotides from chromatogram, and the estimation of nucleotides present in the eluates was exactly the same as described above.

Determination of the nucleotide composition of the RNA fractions: The above eluates from the chromatograms, containing all four nucleotides, were taken to dryness over anhydrous $CaCl_2$ in a vacuum desiccator at room temperature and the residues were taken up in 0.05 *M* ammonium formate buffer at pH 3.4. Aliquots of 0.1 ml were placed as a narrow band at a distance of 10 cm from one end of the strip (9 × 55 cm) of Whatman No. 3 MM filter paper, which was wetted with 0.05 *M* ammonium formate buffer at pH 3.4 and blotted to remove excess buffer. This was subjected to electrophoresis for 3 hr using 0.05 *M* ammonium formate buffer at pH 3.4 and a current of 10 ma. This procedure separates the mixture of nucleotides into four bands in the following order: cytidine 2′ (3′)-phosphate, adenosine 2′(3′−)-phosphate, guanosine 2′(3′−)-phosphate, and uridine 2′(3′)-phosphate. The paper strip was dried at room temperature. The bands were located under ultraviolet light, cut out, and eluted with 0.01 *N* HCl for 18 hr at room temperature. The amount of nucleotide present in each eluate was determined spectrophotometrically.

Growth of tobacco plants in Hoagland's medium containing tritiated uridine: Young tobacco plants having two leaves (1.0 to 1.5 cm long) were removed from sand beds and their roots were washed with tap water. They were placed in bottles containing 50 ml of Hoagland's nutrient medium fortified by the weekly addition of 0.1 ml of 0.5% ferric tartrate. One plant was placed in each bottle covered with black paper to exclude light. The nutrient solution was gently aerated throughout the experimental period. The plants were illuminated 14 hr a day. The temperature of the room was 25°. A week after they were placed in the nutrient medium, 0.2 ml of tritiated uridine (200 μc having a specific activity of 1 curie per mmole) was added to each bottle. The radioactive material was added at four day intervals. During 40 days each plant received 2.0 mc of tritiated uridine. After 40 days the plants were removed from the radioactive medium, their roots were washed under tap water, and they were then placed in bottles containing 200 ml of cold Hoagland's medium.

Infection of radioactive plants with TMV: Immediately after transferring to the cold medium, the leaves that were formed during the growth of the plants in the radioactive medium were retained and the rest were cut out. All the leaves were dusted with carborundum and inoculated with TMV as described above. Ten days after inoculation the leaves were harvested and the radioactivities of the uridylic acid moiety of total RNA, microsomal RNA, and TMV-RNA were determined according to the procedures described below.

Determination of specific activity of uridylic acid in the total RNA of leaf: TCA insoluble residue containing the total RNA of the leaf was obtained and digested with alkali as described above. By employing the chromatographic and electrophoretic techniques described above, uridine 2′(3′−)-phosphate present in the alkali digest of total RNA was isolated. An aliquot of the eluate containing a known amount of uridylic acid was placed in a counting vial and dried *in vacuo* over anhydrous $CaCl_2$ at room temperature. The procedure for measuring the radioactivity is given below.

Determination of specific radioactivities of uridylic acid in microsomal RNA and TMV-RNA: The pellet containing microsomes and TMV, obtained from 2 gm of radioactive leaves, was ex-

tracted with 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA as described above. To this extract (7 ml) was added 400 μg of pancreatic RNAase and a drop of chloroform. As was mentioned above, the extract contains sufficient amount of leaf nuclease to degrade microsomal RNA and pancreatic RNAase was added to hasten this degradation. This was incubated at room temperature for 24 hr.

To 3 ml of the hydrolysate were added 3 ml of chloroform and three drops of octanol. The mixture was shaken at room temperature for 10 min and centrifuged in the clinical centrifuge for 5 min at top speed. The aqueous layer, which was opalescent and colorless, was withdrawn. This contains TMV and ribonucleotides of microsomal RNA. An aliquot was placed on Whatman 3 MM filter paper and dried in a current of air. The chromatogram was developed in isopropanol-water-ammonia solvent system for 24 hr. Under these conditions TMV remains at the origin of the chromatogram and the ribonucleotides separate into two bands: one containing guanylic acid, and the other containing a mixture of adenylic acid, cytidylic acid, and uridylic acid. The second band containing uridylic acid was eluted and the eluate was subjected to electrophoresis as described above. Uridylic acid was eluted from the electrophoretic strip. Uridylic acid and radioactivity in the eluate were determined.

To 3 ml of the ribonuclease digest at 0° was added, with mixing, 0.6 ml of cold 40% TCA. After 15 min, the TCA insoluble precipitate was obtained by centrifugation. The precipitate containing TMV was washed, air-dried, and hydrolyzed with alkali as described above. By employing chromatographic and electrophoretic techniques described above, uridine 2′(3′−)-phosphate present in the alkali digest of TMV was isolated. Uridylic acid and radioactivity in the eluate were determined.

Counting of H^3-uridylic acid: An aliquot of the eluate containing uridylic acid was placed in a counting vial and dried *in vacuo* at room temperature. The dried material was dissolved in 0.5 ml of hyamine. To each vial were added 14.5 ml of scintillation solution containing 40 mg of POPOP and 400 mg of PPO in 100 ml of freshly distilled toluene. The radioactivities were measured in a Packard Tricarb scintillation spectrometer.

Reagents: Pancreatic RNAase was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Uridine-H^3 (specific activity 1 curie per mmole) was obtained from Schwarz Laboratories, Orangeburg, New York. Hyamine 10-x, p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammoniumhydroxide, as a 1.0 *M* solution in methanol, POPOP, 1, 4-bis-2-(5-phenyloxazolyl) benzene and PPO, 2,5-diphenyloxazole were obtained from Packard Instrument Company, La Grange, Illinois.

Results.—Purine and pyrimidine nucleotides of the total, microsomal, and TMV-RNA: In Table 1 are presented the data on the composition of the total, microsomal, and TMV-RNA. The composition was obtained directly on the biological materials without the isolation of RNA in the form of purified preparations, thus eliminating the secondary changes in the composition introduced as a result of the action of nucleases during the isolation. The total RNA of the leaf is derived from the RNA present in all parts of the cell, namely, nucleus, chloroplasts, mitochondria, microsomes, and soluble fraction (soluble RNA); and, as such, the composition given in Table 1 represents the average composition of all the species of RNA present in these subcellular structures. The RNA of the microsomes of the cytoplasmic fraction, which forms about 55–60 per cent of the total RNA of the cell, has a composition similar to that of the total RNA. It is characterized by a high guanylic acid and belongs to the GC-type. The composition of TMV-RNA, on the other hand, is markedly different from that of the total and microsomal RNA. It is characterized by high adenylic and uridylic acids and belongs to the AU-type. Its adenylic acid and uridylic acid contents are higher than those of microsomal RNA by about 37 and 33 per cent, respectively. Thus, the breakdown products of the microsomal RNA can contribute to about 66 per cent of the adenylic acid and uridylic acid needed for the synthesis of TMV-RNA, and the rest, about 34 per cent,

TABLE 1

MOLAR RATIOS OF THE PURINE AND PYRIMIDINE NUCLEOTIDES OF THE TOTAL, MICROSOMAL, AND TMV-RNA*

Nucleotides	Total RNA‡	Microsomal RNA‡	TMV-RNA
Guanylic acid†	1.00	1.00	1.00
Adenylic acid	0.75	0.72	1.15
Cytidylic acid	0.69	0.70	0.73
Uridylic acid	0.70	0.74	1.11

* The values are the averages of four determinations.
† Guanylic acid was arbitrarily taken as 1.00.
‡ Total and microsomal RNA were obtained from healthy tobacco leaves.

must be provided by *de novo* synthesis. Evidence for this mode of TMV-RNA synthesis in the tobacco leaf cell is presented below.

Degradation of microsomal RNA and the utilization of its degradation products in the synthesis of TMV-RNA: The results presented in Figure 1 show the effect of TMV-RNA synthesis on microsomes of the cytoplasmic fraction of the tobacco leaf. Following infection with TMV there was a rapid breakdown of the microsomal RNA while the synthesis of TMV-RNA steadily increased. The amount of TMV-RNA synthesized during the period shown in Figure 1 was approximately equal to the amount of microsomal RNA degraded. Thus, there is a correlation between the synthesis of TMV-RNA and the degradation of the microsomal RNA.

Evidence for the utilization of the breakdown products of the microsomal RNA in the synthesis of TMV-RNA was obtained by analyzing the TMV-RNA formed in the tobacco leaves where RNA was labeled with tritiated uridine. The radioactivities of the uridylic acid of the total, microsomal, and TMV-RNA are given in

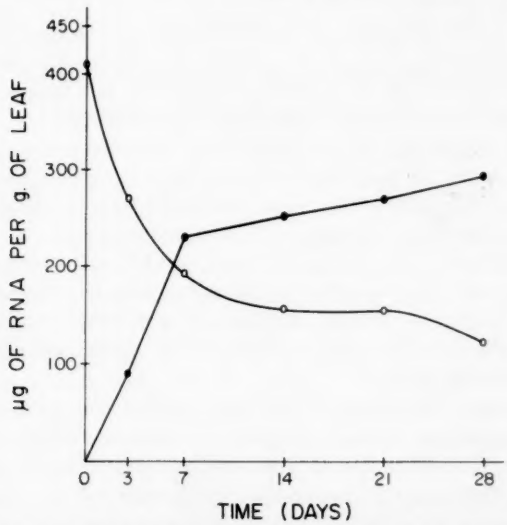

FIG. 1.—Rate of degradation of microsomal RNA during the synthesis of TMV-RNA. O——O, microsomal RNA; ●——●, TMV-RNA. The values are the averages of two determinations performed on two groups of plants grown under identical conditions. Microsomal RNA content of healthy tobacco leaves remained the same throughout the experimental period.

Table 2. The radioactivity of the uridylic acid of TMV-RNA is about 68 per cent of that of the microsomal RNA; i.e., about 68 per cent of the uridylic acid of TMV-RNA was contributed by the microsomal RNA. This amount further correlates with the composition of the microsomal RNA (Table 1). As pointed out above (see section on composition) the nucleotide pool, provided as a result of the breakdown of the microsomal RNA, contains only about 67 per cent of the uridylic acid required for the synthesis of TMV-RNA, and the rest (33 per cent) must therefore be derived by *de novo* synthesis. It is concluded from these results that the microsomal RNA, which forms about 55–60 per cent of the total leaf RNA, acts as a reservoir supplying the ribonucleotides for the synthesis of TMV-RNA.

TABLE 2

SPECIFIC RADIOACTIVITIES OF URIDYLIC ACID OF THE TOTAL AND MICROSOMAL RNA OF THE INFECTED TOBACCO LEAF AND TMV-RNA

Nucleic acids	Counts per min per 50 μg of uridylic acid
Total RNA	1499
Microsomal RNA	1614
TMV-RNA	1096

Discussion.—As shown in this paper, in tobacco leaves infected with TMV, microsomal RNA is rapidly degraded, and its degradation products are utilized in the synthesis of TMV-RNA. The degradation of host nucleic acid and the utilization of its components in the synthesis of viral nucleic acid is not unique to TMV-RNA, since it was shown that the DNA of *Escherichia coli*, infected with T_2 phage, is rapidly degraded to a considerable extent and the degradation products are utilized in the synthesis of T_2 DNA.[8–10]

The degradation of microsomal RNA in the infected tobacco leaf might be effected by the leaf RNAase, the activity of which increases following infection with TMV. The extent of TMV formation parallels the RNAase activity.[11] The increase in RNAase activity could be either due to the removal of an inhibitor or the additional enzyme synthesis. In the case of *E. coli* infected with T_2 phage an apparent increase in the DNAase activity was reported.[12] This increase was later shown to be due to the destruction of a specific DNAase inhibitor (a particular kind of RNA) after infection with phage.[13] We have no evidence for the presence of inhibitor for RNAase in tobacco leaf extracts, and hence the increase in RNAase activity might represent an additional enzyme synthesis. Similar increases in RNAase and a correlation between its activity and virus formation were also reported in bean leaves (*Phaseolus vulgaris* L. var. Pinto and Black Valentine) infected with pod mottle virus.[14]

Summary.—(1) The microsomal RNA present in the cytoplasmic fraction of the tobacco leaf forms about 55-60 per cent of the total leaf RNA. Its purine and pyrimidine composition is similar to that of the total RNA of the leaf. It is characterized by a high guanylic acid and belongs to the GC-type. Its composition is markedly different from that of TMV-RNA which belongs to the AU-type. (2) Following infection with TMV, the microsomal RNA is rapidly degraded and its degradation products are utilized in the synthesis of TMV-RNA. The amount of TMV-RNA synthesized is approximately equal to the amount of microsomal RNA degraded.

Note added in proof: Further evidence was obtained indicating that the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.

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[1] Abbreviations: TMV, tobacco mosaic virus; TMV-RNA, tobacco mosaic virus ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; RNAase, ribonuclease; DNAase, deoxyribonuclease.

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*A LEUCINE ACCEPTOR sRNA WITH AMBIGUOUS CODING PROPERTIES IN POLYNUCLEOTIDE-STIMULATED POLYPEPTIDE SYNTHESIS**

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Weisblum, Benzer, and Holley[1] separated two leucine acceptor sRNAs from *E. coli* by countercurrent distribution. They found that the ribosomal incorporation of leucine attached to one of these is stimulated by poly UC, while leucine attached to the other one responds more readily to poly UG and somewhat to poly U. This experiment proved that leucine is carried to the polynucleotide template by two different molecular species of sRNA having different coding properties. It also provided an explanation for the degeneracy observed in coding experiments with leucine.[2, 3] In this paper it is shown that *E. coli* sRNA can be separated by countercurrent distribution into three leucine acceptors. All three have different coding properties: the first responds preferentially to poly UC, the second responds to poly U and copolymers with a high uridylic acid content, including poly UC 5:1, and the third responds preferentially to poly UG.

Materials and Methods.—Separation of three leucine acceptors: *E. coli* B sRNA was prepared by the method of Zubay.[4] The fraction which precipitates between 0.54 and 0.89 vol of isopropanol was passed over a DEAE cellulose column as described by Holley *et al.*[5] Countercurrent distribution (CCD) was done as described by Apgar *et al.*[6] The first five tubes of a 200 tube apparatus (E. C. Apparatus Co., Philadelphia, Pa.) were charged with 170 mg of *E. coli* sRNA. After 200 transfers, the final fractions were combined into 40 sets of 5 tubes each, reisolated as described, [6] and dialyzed overnight against one change of deionized water. The dialyzed fractions were brought to

dryness in a desiccator over sodium hydroxide pellets and sulfuric acid. Each fraction was dissolved in 2 ml of distilled water and the optical density at 260 mμ was determined.

Assay of leucine acceptor activity: A 100,000 × *g* supernatant of an *E. coli* extract, passed through a Sephadex G-25 column to remove amino acids, was used as the source of the leucine-activating enzyme. The reaction mixture for the assay of the leucine acceptor activity of the sRNA fractions contained in a final volume of 0.5 ml: Tris-HCL, pH 7.2, 50 μmoles; $MgCl_2$, 5 μmoles; KCl, 5 μmoles; adenosine triphosphate, K salt (ATP), 0.5 μmoles; 19 C^{12}-amino acids (leucine omitted), 0.2 μmoles each; 0.1 μc of C^{14}-leucine (specific activity: 10 μc/μmole, New England Nuclear Corporation); reduced glutathione (GSH), 2 μmoles; crystalline beef serum albumin, 100 μg; *E. coli* 100,000 × *g* supernatant, 50 μg protein; and 0.05 ml of the sRNA fraction to be tested. The tubes were incubated for 20 min at 35°C. At the end of the incubation 1 mg of carrier *E. coli* ribosomal RNA was added, and the samples were precipitated and washed with salt-ethanol as described by Berg *et al.*[7] The precipitate was dissolved in dilute ammonia, dried on planchets, and counted in a low background gas-flow counter (Nuclear-Chicago Corporation), with a C^{14}-counting efficiency of 20%.

Preparation of C^{14}-leucyl-sRNA: To remove the sRNA present in the *E. coli* extract used as the source for the leucine-activating enzyme, 10 ml of the 100,000 × *g* supernatant was passed through a 1 × 5 cm column of DEAE cellulose (Serva Entwicklungslabor, Heidelberg), previously equilibrated with 0.1 *M* Tris HCl buffer pH 7.5 containing 0.006 *M* mercaptoethanol.[8,9] The effluent and 10 ml of wash were collected and dialyzed overnight against standard buffer containing 0.01 *M* Tris-HCl pH 7.4, 0.01 *M* Mg Cl_2, 0.06 *M* KCl, 0.006 *M* β-mercaptoethanol. The extract was stored frozen. The reaction mixture contained in a final volume of 2 ml: Tris-HCl pH 7.2 200 μmoles; Mg Cl_2, 20 μmoles; KCl, 20 μmoles; ATP, 5 μmoles; GSH, 8 μmoles; 19 C^{12}-amino acids (leucine omitted), 0.4 μmole each; *E. coli* extract, treated as above, 100 μg protein; C^{14}-leucine (specific activity 246 μc/μmole, New England Nuclear Corporation), 0.04 μmole; sRNA from fraction 14 (Fig. 1, peak 1), 2.6 mg; from fraction 32 (Fig. 1, peak 2), 0.6 mg; from fraction 36 (Fig. 1, peak 3), 0.5 mg, respectively. The three tubes were incubated for 25 min at 35°C, the reaction was stopped by adding 2 ml of redistilled phenol, the tubes were shaken for 10 min at room temperature, and then centrifuged in the cold. The water layer was made 2% with potassium acetate and 0.02 molar with C^{12}-leucine. Then the sRNA was precipitated with 2 vol of ethanol at −20°C; the precipitate was washed once with cold salt-ethanol (see above), dissolved in 2% potassium acetate containing 0.02 *M* leucine, and reprecipitated with 2 vol of ethanol at −20°C. After two more washings with salt-ethanol, the precipitate was dried in a vacuum and the sRNA dissolved in 1 ml of deionized water. In the case of peak 1, 2.4 mg of aminoacyl-sRNA were obtained containing 0.47 × 10^6 cpm per mg sRNA, in the case of peak 2, 0.54 mg sRNA containing 1.3 × 10^6 cpm per mg sRNA, and in the case of peak 3, 0.46 mg sRNA containing 1.04 × 10^6 cpm per mg sRNA.

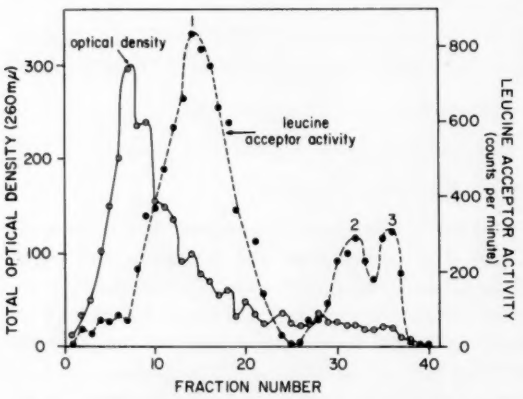

FIG. 1.—Separation of three leucine acceptors by a 200-transfer countercurrent distribution of *E. coli* sRNA (conditions, see text). Each fraction was dissolved in 2 ml of water. The leucine acceptor activity refers to the C^{14}-leucine incorporated into 0.05 ml of each fraction.

Unfractionated *E. coli* sRNA was charged with C^{14}-leucine (specific activity 10 μc/μmole) and reisolated by the method described.[10] It contained 4,000 cpm per mg sRNA. sRNA charged with a mixture of 19 nonradioactive amino acids was prepared in the same way, except that the C^{14}-leucine was omitted.

Preparation of E. coli extract for leucine transfer: Preincubated 30,000 × *g* supernatant of *E. coli* extract (iS-30) was prepared from frozen bacterial paste essentially as described by Nirenberg and Matthaei,[11] except that the cells were broken in a French pressure cell, the 30,000 × *g* supernatant of the extract was preincubated for 45 min at 35°C in standard buffer without any further additions, and, instead of dialysis, the extract was passed over a Sephadex G-50 column. The preparation used had an OD_{260} of 240 per ml. When tested for activity as described,[11] 250-fold stimulation of incorporation of free C^{14}-phenylalanine was produced by 15 μg of poly U.

Transfer of C^{14}-leucine bound to sRNA into polypeptide: The reaction mixture contained in a final volume of 1 ml: Tris-HCl pH 7.6, 100 μmoles; $MgCl_2$, 15 μmoles; K Cl, 50 μmoles; guanosine triphosphate, 0.05 μmole; phosphoenolpyruvate K salt, 7 μmoles; pyruvate kinase, 20 μg; β-mercaptoethanol, 6 μmoles; 19 C^{12}-amino acids, 0.2 μmoles each; C^{12}-leucine, 2 μmoles; C^{12}-aminoacyl-sRNA (leucine omitted), 0.2 mg; C^{14}-leucyl-sRNA as stated in the legends of Figures 2, 3, 4, and 5; 5 μg poly U, poly UG 5:1, poly UA 5:1, poly UC 5:1, or poly UC 1:1 as indicated; iS-30, 0.1 ml.

The mixture was incubated at 35°C. At the times indicated, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). The RNA was hydrolyzed for 15 min in a boiling water bath and the precipitate was collected and washed with 5% TCA, containing carrier leucine, on a Millipore filter. The filters were glued on planchets, dried, and counted. The amount of amino acid transferred was proportional to the aminoacyl-sRNA added.

Synthetic polyribonucleotides: Poly UC (input ratio 1:1) and poly UG (input ratio 5:1) were kindly prepared for us by Dr. R. F. Beers. Poly UA (input ratio 5:1) and poly UC (input ratio 5:1) were kindly donated by Dr. B. Weisblum. Poly U was purchased from the Miles Chemical Company, Clifton, New Jersey.

Results.—Separation of three leucine acceptors: The results of a 200-transfer countercurrent distribution of *E. coli* sRNA are shown in Figure 1. The recovery of ultraviolet-absorbing material was 75 per cent. The assay of leucine acceptor activity shows three peaks, one broad peak with the maximum at fraction 14, which is clearly separated from a double peak, moving almost with the front, with maxima at fractions 32 and 36. Samples taken from these fractions will subsequently be referred to as peak 1, peak 2, and peak 3 sRNA.

Coding properties of peak 1, peak 2, and peak 3 sRNA: Aliquots of fractions 14, 32, and 36 were charged with C^{14}-leucine, and their response to different polymers was determined. Poly UA 5:1 and poly UC 5:1 were tested in addition to the polymers used by Weisblum *et al.*,[1] poly UC 1:1, poly UG 5:1, and poly U.

The kinetics of transfer of C^{14}-leucine from peak 1 sRNA in response to these 5 polymers is shown in Figure 2. Both poly UC 1:1 and poly UC 5:1 are active; poly UC 5:1 produces the highest level of stimulation. Although the response of peak 1 sRNA to poly UA and poly UG is small, it nevertheless is significant because it was obtained repeatedly. Thus, the coding properties of our peak 1 sRNA are similar to the coding properties reported by Weisblum *et al.*[1] for their peak 1 sRNA.

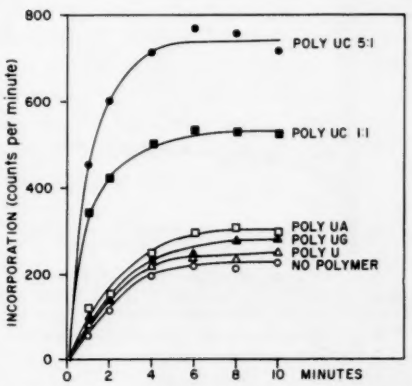

FIG. 2.—Polynucleotide-dependent incorporation of leucine bound to peak 1 sRNA. 0.013 mg C^{14}-leucyl peak 1 sRNA were added, containing 6,000 cpm (conditions as described in *Methods*). The input ratio of nucleotides in the synthesis of the copolymers was 5:1 unless noted otherwise.

The response of peak 2 sRNA to the polymers is shown in Figure 3. In this case all polymers with a high uridylic acid content stimulated the transfer of C^{14}-leucine; poly UG and poly U were the most active. It should be noted that the UC copolymer, which was found inactive with the peak 2 sRNA by Weisblum *et al.*,[1] was the poly UC 1:1. In agreement with their result, poly UC 1:1 had only a small but significant effect. The poly UC 5:1, on the other hand, was almost as active as the other copolymers containing a high proportion of uridylic acid. It is concluded therefore that the coding properties of this leucine acceptor sRNA are very similar to the coding properties that have been reported for the phenylalanine acceptor sRNA.[3, 11, 12]

Figure 4 shows the response of peak 3 sRNA to the 5 polymers. Poly UG has by far the greatest effect, whereas poly UA, poly UC 5:1, and poly U have dropped considerably.

The relatively high level of stimulation by poly UG in the case of peak 2 sRNA, and the still significant stimulation by poly UA, poly U, and poly UC 5:1, in the case of peak 3 sRNA, is probably due to the incomplete separation of peaks 2 and 3 sRNA. It is very likely that the peak 2 sRNA of Weisblum *et al.*[1] contained a mixture of our peak 2 and peak 3 sRNA.

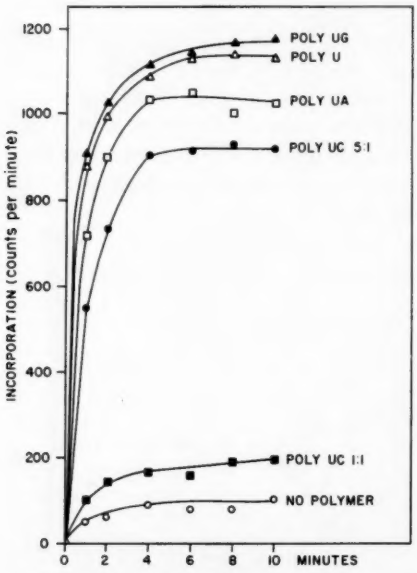

FIG. 3.—Polynucleotide-dependent incorporation of leucine bound to peak 2 sRNA. 0.006 mg C^{14}-leucyl peak 2 sRNA were added, containing 7,900 cpm (conditions as described in *Methods* and in Fig. 2).

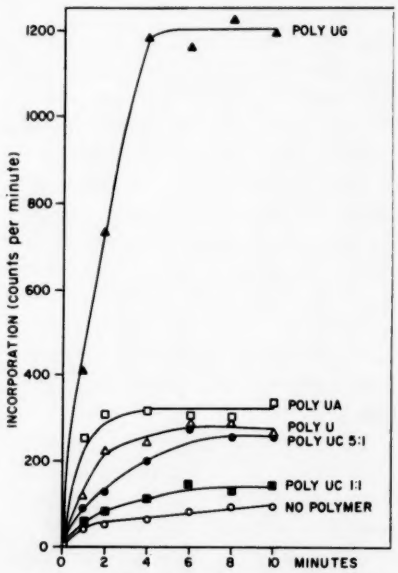

FIG. 4.—Polynucleotide-dependent incorporation of leucine bound to peak 3 sRNA. 0.005 mg C^{14}-leucyl peak 3 sRNA were added, containing 5,200 cpm (conditions as described in *Methods* and in Fig. 2).

The pattern of transfer of C^{14}-leucine charged to unfractionated sRNA in response to the various polymers is shown in Figure 5. The relative abundance of peak 1 sRNA versus peak 2 and peak 3 sRNA in the unfractionated mixture is reflected by the relatively large effect of poly UC 1:1 and also of poly UC 5:1. It should be

kept in mind that both peak 1 sRNA and peak 2 sRNA respond to poly UC 5:1.

Discussion.—The results support the conclusion by Weisblum *et al.*[1] that degeneracy in the case of leucine can be accounted for by the different coding properties of different acceptors. As has been pointed out by the same authors, two kinds of degeneracy are possible. In the first kind more than one coding unit specifies one amino acid; in the second kind one coding unit specifies more than one amino acid, and in this case the coding unit is said to be ambiguous. The existence of ambiguity in the amino acid code was suggested by the observation that both phenylalanine and leucine are incorporated by *E. coli* ribosomes in response to poly U.[1, 13, 14] The experiment described in this paper shows that leucine is carried to the template by a separate leucine acceptor which has ambiguous coding properties.

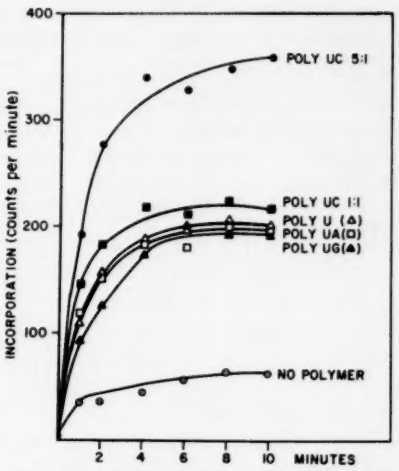

FIG. 5.—Polynucleotide-dependent incorporation of leucine bound to unfractionated *E. coli* sRNA. 1.0 mg of C^{14}-leucyl-sRNA were added, containing 4,000 cpm. The specific activity of the C^{14}-leucine was lower (10 $\mu c/\mu mole$) than that used in Figs. 2, 3, and 4 (conditions as described in *Methods* and in Fig. 2).

Whether the results obtained with the artificial polymers also apply to the synthesis of natural proteins is currently being tested by transferring leucine bound to these three acceptors into the leucine positions of hemoglobin in a ribosomal system from rabbit reticulocytes,[10] and into the leucine positions of the coat protein of the MS_2-bacteriophage utilizing the system described by Nathans *et al.*[15]

Summary.—*E. coli* contains three leucine acceptor sRNAs separable by countercurrent distribution. Transfer into polypeptide of leucine attached to the first one is stimulated preferentially by poly UC, leucine attached to the second one is transferred in response to polynucleotides containing a high proportion of uridylic acid including poly U, and leucine attached to the third acceptor is incorporated preferentially in response to poly UG. These results provide an explanation for the ambiguity in coding for leucine observed in polynucleotide-dependent polypeptide synthesis.

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*PROTEIN SYNTHESIS BY RETICULOCYTE RIBOSOMES, I. INHIBITION OF POLYURIDYLIC ACID-INDUCED RIBOSOMAL PROTEIN SYNTHESIS BY CHLORAMPHENICOL**

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Chloramphenicol is known to inhibit microbial protein synthesis both in intact cells[1–4] and in cell-free systems.[5, 6] Similar inhibition of protein synthesis has not, however, been demonstrable in analogous mammalian systems.[7–9] Matthaei and Nirenberg observed inhibition of protein synthesis by *E. coli* ribosomes with 0.3 μmoles of chloramphenicol per ml reaction mixture.[5] However, von Ehrenstein and Lipmann were unable to obtain an appreciable inhibition in mammalian cell-free systems with 0.01 molar amounts of chloramphenicol.[7] Similarly, inhibition of amino acid incorporation into intact reticulocytes *in vitro* has been noted only with amounts of chloramphenicol which greatly exceed pharmacologic concentrations.[9]

Although mammalian ribosomes do not appear to be affected by chloramphenicol, there is considerable evidence that chloramphenicol can exert deleterious effects in man. Cells of the hematopoietic system appear to be particularly susceptible as manifested by the occasional development of anemia, leukopenia, thrombocytopenia, or aplastic anemia during chloramphenicol therapy.[10, 11] Although the mechanism of toxicity is unknown, certain clinical observations suggest that chloramphenicol is most likely to affect primitive or immature cells during a stimulatory or maturation phase. Thus, Saidi *et al.*[12] observed that patients with pernicious anemia in relapse who were receiving chloramphenicol at the time when B_{12} therapy was initiated, failed to manifest the reticulocyte response seen as a result of this type of therapy. Furthermore, the characteristic reticulocyte response did not occur in these patients until after chloramphenicol was discontinued. In such patients normoblastic maturation of the megaloblastic marrow occurred despite the inhibition of reticulocytosis, indicating that purine and pyrimidine biosynthesis was unaffected. This is analogous to the sequence of events in bacteria where chloramphenicol inhibits protein synthesis without inhibiting DNA or RNA synthesis.[1]

These observations suggest that megaloblasts may be uniquely susceptible to chloramphenicol inhibition, whereas cells at a later stage of maturation remain un-

affected. Presumably the initial steps of maturation would require the synthesis of ribosomal template RNA capable of directing the synthesis of hemoglobin and other proteins necessary for cell development. Marks *et al.*[13] demonstrated that little or no new formation of ribosomal RNA occurs in reticulocytes capable of protein synthesis. The possibility therefore exists that chloramphenicol may exert an inhibitory effect before or during the deposition of "messenger" or template RNA in primitive hematopoietic cells and that once this template has been formed on the ribosome, the cell is no longer susceptible to chloramphenicol inhibition.

Although the intact reticulocyte has no mechanism for the synthesis of new template RNA, it is possible to stimulate protein synthesis by reticulocyte ribosomes in cell-free systems with polyuridylic acid (poly U), a synthetic polyribonucleotide which functions as template or "messenger" RNA in directing the incorporation of phenylalanine into polypeptide chains.[14, 15] Since this essentially represents the deposition of new template RNA on the ribosome, the possibility of inhibiting poly U-induced ribosomal protein synthesis by chloramphenicol was investigated. Under the conditions of the experiments to be reported, inhibition of protein synthesis by ribosomes was obtained with as little as 0.001 μmole of chloramphenicol per ml reaction mixture. This inhibition appeared to be due to an effect of chloramphenicol on uridylic acid residues of poly U, resulting in either inactivation of the template or in preventing its deposition on the ribosome. It is not possible to state from these experiments whether chloramphenicol can affect all "messenger" RNA in a similar manner or whether it has a specific affinity for uridylic acid residues of poly U.

Material and Methods.—The cell-free systems used in these studies were modifications of those employed by Matthaei and Nirenberg[5] and Allen and Schweet[8] and consisted essentially of rabbit reticulocyte ribosomes, supernatant factors obtained from either reticulocytes or *E. coli*, an energy generating system, amino acids, and buffer in a final volume of 1 ml.

Preparation of ribosomes: Reticulocytosis was produced in New Zealand rabbits with phenylhydrazine by the method of Borsook *et al.*,[16] and ribosomes were prepared by a modification of the method of Allen and Schweet.[8] Blood with a reticulocyte count of 85–95% was obtained by cardiac puncture and washed with 0.13 *M* NaCl, 0.005 *M* KCl and 0.0075 *M* $MgCl_2$. The packed cells were lysed with 4 volumes of 0.002 *M* $MgCl_2$, stirred at 4°C for 10 min, after which 1 vol. of 1.5 *M* sucrose containing 0.15 *M* KCl was added. The mixture was stirred for 20 min and centrifuged at 15,000 × *g* for 30 min at 4°C. The sediment was discarded, and the supernate was centrifuged for 2 hr at 105,000 × *g* in a preparative ultracentrifuge at 4°C. The supernatant fluid was subsequently used to prepare pH 5 enzyme.[17] The ribosomal pellet was rinsed and resuspended in 0.002 *M* $MgCl_2$, 0.0175 *M* $KHCO_3$, and 0.25 *M* sucrose to the original volume, and then centrifuged at 4°C for 1 hr at 105,000 × *g*. This supernatant was discarded and the pellet rinsed with 0.25 *M* sucrose. The ribosomal pellet was then resuspended in $^1/_2$ the original volume of packed cells by gentle homogenization with 0.25 *M* sucrose in a loose fitting glass homogenizer. The ribosomal suspension was centrifuged at 15,000 × *g* for 5 min, the sediment discarded, and aliquots of the supernate were stored at −70°C. This preparation retained activity for at least 4 weeks.

Preparation of supernatant factors: The pH 5 enzyme was prepared from reticulocytes by a modification of the method of Keller and Zamecnik,[17] using the supernatant obtained after centrifuging the lysed reticulocytes for 2 hr at 105,000 × *g*. This supernate contained large amounts of hemoglobin, and the procedure to be described removed all but traces of hemoglobin and other interfering substances. Three volumes of 0.9 *M* sucrose, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl (Medium B of Keller and Zamecnik) were added to the reticulocyte supernate and the pH adjusted to 5.2 by slowly adding 1 *N* acetic acid. All solutions were kept at 4°C during this and subsequent preparative procedures. The precipitate was recovered by centrifugation at 10,000 × *g* for 15 min, washed, and then resuspended by homogenization with 40–50 ml of Medium B.

The protein pellet was again collected by centrifugation at 30,000 × *g* for 10 min. The pellet was rinsed with 0.35 *M* sucrose, 0.035 *M* $KHCO_3$, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl (Medium A of Keller and Zamecnik) and then dissolved in this same solution by homogenization. The final volume was adjusted to half the original packed cell volume. Insoluble protein was removed by centrifugation at 30,000 × *g* for 10 min. The supernatant solution containing approximately 4–5 mg of protein per ml was stored in aliquots at −70°C and retained activity for more than 4 weeks.

A supernatant fraction (S100) was obtained from *E. coli* K12 λ harvested in early log phase, by the methods of Matthaei and Nirenberg.[5] Aliquots were stored at −70°C and retained activity for more than 4 weeks.

Protein determinations were made by the Folin-phenol method of Lowry,[18] and RNA content was estimated by the orcinol method,[19] using d-ribose as a standard. The protein concentrations in the pH 5 enzyme preparations ranged between 2.9 and 4.0 mg per ml, and in the S100 fractions ranged between 3.85 and 4.4 mg per ml. The pH 5 enzyme contained 0.25 mg of RNA per ml as compared to 0.30 mg per ml in the S100 fraction. The S100 fraction contained the equivalent of 2.5 μgm per ml of RNAase activity,[20] whereas no nuclease activity was demonstrable in the pH 5 enzyme fraction.

Reaction mixtures and assay techniques: The components of the reaction mixtures were essentially those employed by Matthaei and Nirenberg.[5] Each reaction mixture contained 100 μmoles of Tris (hydroxymethyl) aminomethane, pH 7.8; 10 μmoles magnesium acetate; 50 μmoles KCl; 6 μmoles mercaptoethanol; 0.05 μmoles of 20 L-amino acids;[9] 0.01–0.025 μmoles U-C^{14}-L-phenylalanine (600,000–1,200,000 c/m); 0.025 μmoles GTP; 0.025 μmoles CTP; 0.025 μmoles UTP; 5 μmoles phosphoenolpyruvate (PEP); 1 μmole K_2 ATP; 20.0 mg of pyruvate kinase; 0.2–0.25 mg of ribosomal protein, and 0.2–0.4 mg of either pH 5 or S100 protein. When C^{14}-L-leucine incorporation was investigated, C^{12}-L-leucine was omitted from the amino acid mixture, and 0.002 μmoles of C^{14}-L-leucine (125,000 c/m) were added to the reaction. All components were added at 4°C, and the final volume adjusted to 1.0 ml with water. The reaction mixtures were incubated at 37°C for 1 hr, and the reaction was terminated by the addition of 2 ml of 5% trichloroacetic acid (TCA). The protein precipitate was washed with 2 ml of 5% TCA and then extracted with 5% TCA at 90°C for 20 min. The precipitate was again washed with 5% TCA, washed once with ethanol-ether (3:1), and then with anhydrous ether. The residue was dried, dissolved in 1.3 ml of concentrated formic acid, and 1.0 ml aliquots were counted in a liquid scintillation counter (Nuclear-Chicago).

Materials: 2-Phosphoenolpyruvic acid (crystalline tricyclohexylammonium salt) and pyruvate kinase (muscle) A grade, specific activity 168–254 E.U./mg protein were obtained from either California Corporation for Biochemical Research or Sigma Chemical Company. ATP (potassium salt) was obtained from Nutritional Biochemical Corporation. GTP, UTP, and CTP were obtained from California Corporation for Biochemical Research. Uniformly labeled C^{14}-L-phenylalanine, specific activity 50 mc per mM, was obtained from Nuclear-Chicago. U-C^{14}-L-leucine, specific activity 110 mc per mM, was obtained from Schwarz BioResearch, Inc. Crystalline chloramphenicol was supplied through the courtesy of Parke-Davis & Co. Polyuridylic acid (potassium salt) was furnished initially by Dr. Oliver Jones of the National Institutes of Health and was subsequently obtained from Miles Chemical Co., Elkhart, Indiana.

Results.—The requirements for protein synthesis by reticulocyte ribosomes in the system employed were identical with those previously reported by others using similar reaction mixtures.[5–7] Omission of any of the essential components such as the energy generating system, supernatant factors, amino acid mixture, or ribosomes resulted in absent or markedly reduced incorporation of C^{14}-L-leucine into TCA precipitable protein. Protein formation was directly proportional to the amount of ribosomes added and was also influenced by the amount of supernatant factor added. Chloramphenicol, in a concentration of 2 μmoles per ml of reaction mixture, did not appreciably inhibit the incorporation of C^{14}-L-leucine, the maximum inhibition being less than 5 per cent.

Poly U stimulation of C^{14}-L-phenylalanine incorporation: Poly U stimulated the

incorporation of C^{14}-L-phenylalanine in the ribosomal system both with *E. coli* supernate (S100) and with reticulocyte pH 5 enzyme as a source of sRNA, amino acid activating enzyme, and transfer enzyme. The pH 5 enzyme was 10 to 25 times more effective per mg of protein than the S100 supernate in promoting the formation of polyphenylalanine in the presence of poly U (Fig. 1). Similar results were obtained with both freshly prepared or frozen pH 5 and S100 fractions. When both supernatant factors were used in the reaction mixture, the amount of C^{14}-L-phenylalanine incorporated was not additive and was less than the mean value (Fig. 1). Maximum amounts of phenylalanine incorporation were obtained with 25–50 μg of poly U with either pH 5 enzyme or S100 supernate.

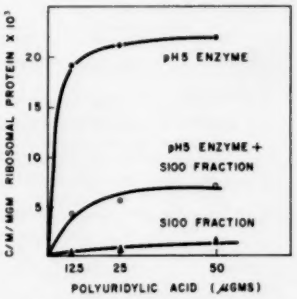

FIG. 1.—Comparative effects of pH 5 enzyme and S100 supernatant factor on ribosomal protein formation stimulated by poly U. Components of the reaction mixture are described in *Methods*. The S100 fraction contained 0.19 mg of protein, and the pH 5 enzyme contained 0.4 mg of protein. 0.02 μM of C^{14}-L-phenylalanine $\sim$ 1,200,000 c/m was present in each reaction mixture.

Although the reticulocyte pH 5 enzyme was more effective than the *E. coli* S100 supernate in the C^{14}-L-phenylalanine-poly U system, their relative effectiveness in promoting the incorporation of C^{14}-L-leucine into TCA precipitable protein was reversed, the S100 fraction being 2 to 3 times as effective as the pH 5 enzyme.

Inhibition of poly U stimulation by chloramphenicol: Chloramphenicol inhibited the formation of C^{14}-labeled polyphenylalanine induced by poly U. At the completion of reaction, the percentage of inhibition by a given concentration of chloramphenicol was inversely proportional to the amount of poly U used to stimulate phenylalanine incorporation, the most marked inhibition being evident with small amounts of poly U.

Partial inhibition of the stimulation obtained with 12.5–25 μg of poly U was observed with 0.5 μmoles of chloramphenicol per ml when S100 was used as the supernatant factor (Fig. 2). Maximal inhibition was obtained with 2–3 μmoles of chloramphenicol, the most marked inhibition being observed with 12.5 μg of poly U which was the smallest amount tested in the experiments with the S100 fraction (Table 1).

TABLE 1

INHIBITION BY CHLORAMPHENICOL OF POLY U EFFECT ON RETICULOCYTE RIBOSOMES

Amount of poly U	Supernatant factor	Incorporation of C^{14}-Phenylalanine (% of control) Chloramphenicol 0.005μM	Chloramphenicol 0.5μM	Chloramphenicol 1.0μM
50 μg	S100	100	81	76
25 "	S100	100	82	72
12.5 "	S100	100	75	31
5.0 "	pH 5 Enz.	94	75	76
2.5 "	pH 5 Enz.	84	35	40
1.0 "	pH 5 Enz.	52	6	0

The sensitivity of the system to chloramphenicol inhibition was greater when pH 5 enzyme was used instead of S100 as the supernatant factor. The most effective inhibition with chloramphenicol was obtained in reaction mixtures containing pH 5 enzyme and 1 μg of poly U. In such reaction mixtures an inhibitory effect

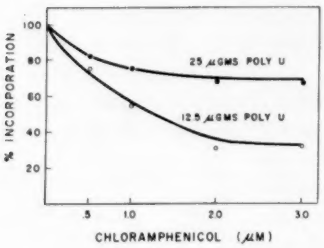

FIG. 2.—The effect of chloramphenicol on polyphenylalanine incorporation stimulated by poly U using S100 supernatant factor. Components of the reaction mixture are stated in *Methods*. Each assay contained 0.38 mg of S100 protein and 0.01 μM of C^{14}-phenylalanine ~ 600,000 c/m. The inhibitory effect of chloramphenicol obtained with 12.5 μg of poly U was compared with that obtained when 25 μg of poly U was used to stimulate the ribosomes. Greater inhibition was obtained with equivalent amounts of chloramphenicol when 12.5 μg of poly U were used.

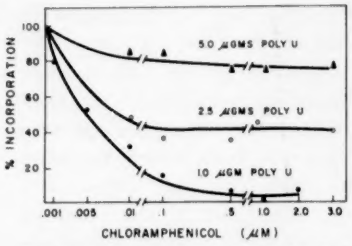

FIG. 3.—The effect of chloramphenicol on phenylalanine incorporation stimulated by poly U using reticulocyte pH 5 enzyme as a supernatant factor. Components of the reaction mixture are stated in *Methods*. Each assay contained pH 5 enzyme (0.4 mg of protein) and 0.02 μM of C^{14}-phenylalanine ~ 1,200,000 c/m. The inhibitory effect of chloramphenicol was determined in relation to the stimulation obtained with 1.0, 2.5, and 5.0 μg of poly U. The most effective inhibition was obtained when 1.0 μg of poly U was used. An inhibitory effect was detectable with as little as 0.001 μM of chloramphenicol in relation to 1.0 μg poly U (0.003 μM of uridylic acid residues).

was detectable with as little as 0.001 μM of chloramphenicol per ml, and 100 per cent inhibition was obtainable with 1.0 μM of chloramphenicol per ml (Table 1, Fig. 3). No inhibition was obtained with less than .001 μM of chloramphenicol per ml. Chloramphenicol inhibition was less effective when the ribosomes were stimulated with 2.5 μg or 5.0 μg of poly U.

Inhibition of the ribosomal protein formation induced by poly U was dependent upon the presence of chloramphenicol in the reaction mixture before the poly U could react with ribosomes and phenylalanine. No inhibition could be obtained if poly U was incubated with ribosomes in the presence of phenylalanine for 5 min prior to adding chloramphenicol (Table 2). This lack of chloramphenicol effect occurred despite the fact that no protein formation took place during this 5 min incubation period.[24] The maximum inhibitory effect occurred when both poly U and chloramphenicol were present in the reaction mixture prior to the addition of ribosomes.

TABLE 2

THE EFFECT OF CHLORAMPHENICOL ON RIBOSOMES PREINCUBATED WITH POLY U

Complete system:	Net C/M/mg ribosomal protein	% Incorporation
+ 1 μg poly U	125	100
+ μg poly U + 1 μM chloramphenicol (no preincubation)	0	0
+ 1 μg poly U + 1 μM chloramphenicol after 5 min incubation	155	120
+ 2.5 μg poly U	450	100
+ 2.5 μg poly U + 1 μM chloramphenicol (no preincubation)	315	70
+ 2.5 μg poly U + 1 μM chloramphenicol after 5 min incubation	490	108
+ 5.0 μg poly U	1.550	100
+ 5.0 μg poly U + 1 μM chloramphenicol (no preincubation)	1,150	74
+ 5.0 μg poly U + 1 μM chloramphenicol after 5 min incubation	1,625	105

Discussion.—These results indicate that, under certain conditions, chloramphenicol can exert an inhibitory effect on protein synthesis by reticulocyte ribosomes. Inhibition occurred only in the presence of added "messenger" or template RNA and could be readily overcome by adding an excess of poly U. Matthaei and Nirenberg also observed chloramphenicol inhibition of poly U stimulation, but their studies are carried out on *bacterial* ribosomes.[6] Since chloramphenicol readily inhibits microbial ribosomal protein synthesis, it is not possible to attribute the inhibition observed in such a system to an effect of chloramphenicol on poly U. In contrast, the reticulocyte ribosome is known to be unaffected by chloramphenicol under ordinary circumstances, and the inhibition observed in the present studies can be attributed to an effect of chloramphenicol on poly U. Chloramphenicol did not inhibit phenylalanine incorporation stimulated by poly U after it was incubated with ribosomes for a brief interval, during which time the poly U presumably becomes attached to the ribosome and interacts with activated sRNA-phenylalanine. Chloramphenicol may therefore either prevent the deposition of poly U on ribosomes or combine with or inactivate the uridylic acid residues before they can react with the activated sRNA-phenylalanine. It is not known from these experiments whether chloramphenicol can exert a similar inhibitory effect on any new "messenger" or template RNA, or whether its effect is specific for poly U. Preliminary results indicate that chloramphenicol can inhibit viral and other RNA-directed ribosomal protein synthesis.[21] It remains to be determined whether chloramphenicol inhibition is specifically related to an effect on uridylic acid residues of poly U or whether it can inhibit other ribonucleotides. The ability of chloramphenicol to inhibit various types of "messenger" or template RNA is currently being investigated.

The incorporation of phenylalanine induced by 1 μg of poly U (0.003 μmoles of uridylic acid residues per ml) was partially inhibited by as little as 0.001 μmole of chloramphenicol per ml. This suggests that an inhibitory effect may become manifest when chloramphenicol combines with or inactivates an average of 1 of 3 uridylic acid residues of poly U at random. Since the code for phenylalanine is presumably a triplet of uridylic acid, inactivation of any one of three consecutive uridylic acid residues could cause decreased formation of polyphenylalanine. This would account for the decreased inhibition observed with 2.5 μg (0.0075 μM uridylic acid residues) and 5.0 μg (0.015 μM uridylic acid residues) of poly U per ml when exposed to comparable amounts of chloramphenicol, as well as the lack of an inhibitory effect when excessive amounts of poly U are added to the system. The attainment of a limiting value of inhibition when 2–3 μM of chloramphenicol (Figs. 2 and 3) were used in the reaction mixtures may be due to saturation of available binding sites. Another factor which would contribute to the plateau is that the same amount of inhibition of polyphenylalanine formation would be expected whether 1, 2, or 3 consecutive uridylic acid residues of poly U were inactivated. Presumably, inhibition should be observed even with large amounts of poly U if sufficient chloramphenicol were present to combine in a random fashion with a critical number of uridylic acid residues. Such studies were not possible because of the limited solubility of crystalline chloramphenicol.

The marked differences in polyphenylalanine formation observed with S100 and pH 5 enzyme supernatant factors may be due to the presence of ribonuclease in

the former causing degradation of poly U. No ribonuclease activity was detected in the pH 5 enzyme fraction. Accordingly, the assay with pH 5 enzyme is a more reliable index of the inhibitory effect of chloramphenicol relative to poly U.

The ability of chloramphenicol to prevent the incorporation of amino acids by synthetic "messenger" RNA would appear to correlate with clinical observations on the inhibitory effect of the drug during therapy of megaloblastic anemias, namely, during that stage of development when the primitive erythrocyte is rapidly synthesizing messenger RNA. It is also possible to explain the occurrence of hematologic toxicity following chloramphenicol administration under other circumstances.[25]

These experiments do not elucidate the fundamental difference in the sensitivity of bacterial and mammalian ribosomes to chloramphenicol. Although it has been assumed that the mechanism of protein synthesis is similar in all cells, there may be a fundamental difference in the microbial ribosomes as suggested by von Ehrenstein and Lipmann.[7] Conceivably reactive sites on microbial template RNA may be more accessible for combination with chloramphenicol.

Summary.—An inhibitory effect of chloramphenicol on protein synthesis by rabbit reticulocyte ribosomes is described. This inhibition occurred only in the presence of added "messenger" or template RNA. The possibility that chloramphenicol may inactivate template RNA is discussed.

We wish to thank Dr. Lazarus Astrachan and Dr. Melvin D. Schoenberg for valuable advice, suggestions, and assistance in performing these experiments and to express our appreciation to Dr. Oliver Jones of the National Institutes of Health for supplying us with the initial samples of polyuridylic acid. We also wish to acknowledge the technical assistance of E. Ceasar Moss.

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[25] Such postulation requires the assumption that 0.001 μM of chloramphenicol in a cell-free system is equivalent to toxic levels found in the serum (0.05 to 0.1 μM per ml)[22, 23] and that the amount of "messenger" RNA present in maturing hematopoietic cells is less than the equivalent of 1 μg of poly U. Although these are reasonable assumptions, it is quite probable that chloramphenicol has other toxic effects on cellular metabolism as well.

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*THE NATURE OF THE RNA RESPONSE TO ESTRADIOL ADMINISTRATION BY THE UTERUS OF THE RAT**

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Over the past several years abundant evidence has accrued to indicate that the administration of sex hormones results in an increase both in the content[2–6] and in the rate of synthesis[7] of ribonucleic acid (RNA) in the accessory sex tissues. Furthermore, although evidence has been presented to the contrary,[8] several kinds of studies have indicated that the enhancement of protein synthesis by these hormones is secondary to the increased rate of RNA synthesis. First, the biochemical site of action of testosterone on protein synthesis in rat seminal vesicle and of estradiol on protein synthesis in hen oviduct has been localized to the RNA-rich microsomes.[9–11] Second, inhibition of protein synthesis does not interfere with the estrogen-induced acceleration of RNA synthesis.[12] Finally, the addition of synthetic polyribonucleotides has been shown to accelerate the synthesis of protein in homogenates of control but not of testosterone-treated prostate.[13]

The implication that the acceleration of protein synthesis following hormone administration is secondary to enhanced RNA synthesis does not of itself, however, explain the mechanism by which protein synthesis is accelerated. At least three different types of RNA are involved in protein synthesis—soluble or transfer RNA, ribosomal structural RNA, and messenger or template RNA. That transfer RNA is not rate-limiting for protein synthesis in these hormone-responsive tissues has been suggested by two types of experiments—measurements of transfer C^{14}-RNA-amino acids in slices of rat seminal vesicle,[9] and the demonstration that no soluble factors appear to be rate-limiting in protein synthesis by homogenates of hen oviduct.[10, 11] Furthermore, the studies of Liao and Williams-Ashman indicate that template RNA is rate-limiting for protein synthesis in the prostate of the castrated rat.[13]

The present communication describes studies on the sequence and nature of the RNA response to estradiol administration by the immature rat uterus. Evidence is presented that the administration of estradiol initially enhances the synthesis of transfer RNA and of template RNA, and that enhanced synthesis of ribosomal structural RNA is a late effect.

Materials and Methods.—Female Sprague-Dawley rats, weighing 100–150 gm, were used for the experiments. Oophorectomies were performed under ether anesthesia; 10–20 days following the operation the animals were given by injection into the tail vein either 10 μg of estradiol-17β dissolved in 1 ml of 0.9% saline or the saline alone. The animals were killed by decapitation, and the uteri were excised and immediately chilled. For each experiment the tissues from 20 to 50 animals were pooled. The subscripts E and C refer to the source—either estradiol-treated or control uterus—of the fractions studied.

Incubation and extraction techniques: The chilled tissues were sliced with a McIlwain tissue slicer, blotted, weighed, and transferred to incubation tubes containing 4×10^{-4} *M* adenine-8-C^{14} (2.1×10^6 cpm), 6×10^{-3} *M* glucose, and Krebs-Ringer-bicarbonate buffer, pH 7.4, to make a final volume of one ml. After gassing with 95% oxygen–5% carbon dioxide, the tubes were capped and incubated in one of two ways. In the pulse experiments the incubations were stopped after 30 min; in the chase experiments one μmole of adenine was added to the tubes after 30 min, and the incubation was continued for three hr. Following incubation 10 ml of the cold Krebs-Ringer-bicarbonate buffer was added, and the tubes were centrifuged for 30 seconds.

An RNA extraction procedure similar to that described by Perry[14] was used. The pellets were suspended at 0° in 2 ml of 0.01 *M* acetate buffer, pH 5.0, containing 0.5% recrystallized sodium dodecyl sulfate, 0.1 *M* NaCl, and 10^{-3} *M* $MgCl_2$. The extracts were shaken for 3 min at 60°C with 3 ml of freshly distilled, water-saturated phenol, quickly chilled, and centrifuged. The aqueous phase was removed and extracted twice more with phenol at 60°C. The final extract was shaken five times with ether to remove the phenol, and the RNA was precipitated with 2 volumes of ethanol at −20°C. The RNA precipitate was dissolved in 2 ml of acetate-NaCl-$MgCl_2$ buffer and passed through a Sephadex G25 column (1 × 20 cm). The RNA was eluted with 4–9 ml of the buffer and again precipitated at −20°C with 2 volumes of ethanol. In experiments in which the messenger activity was assayed, the RNA was dialyzed in the cold for 2–6 hr against 100 volumes of acetate-NaCl-$MgCl_2$ buffer containing 0.1% mercaptoethanol and again precipitated at −20°C with ethanol. The phenol extraction procedure of Kirby,[15] as described by Hoagland *et al.*,[16] was used in some experiments; no difference was noted between the two procedures either in the centrifugation pattern or in the messenger activity of the final RNA preparation.

The RNA was then dissolved in 0.2 ml of the acetate-NaCl-$MgCl_2$ buffer and applied to a 5–20% sucrose gradient according to the method of Britten and Roberts.[17] The preparations were centrifuged at 27,000 rpm for 8–11 hr at 5°C in a Spinco SW 39 swinging bucket rotor. After completion of the centrifugation a hole was punched in the bottom of each tube, and 10 or 20 drop fractions were collected as described by Szybalski.[18] Absorbances at 260 mμ were determined, and portions of each sample were added to 10 ml of the scintillation liquid described by Bray[19] and assayed for C^{14} in a Packard liquid scintillation counter. In experiments in which individual peaks of RNA were assayed for messenger activity several fractions were combined, precipitated with ethanol as before, dried under nitrogen, dissolved in 0.1 ml of buffer, and assayed in the amino acid incorporating system.

Preparation of amino acid incorporation system: The system employed was essentially that of Keller and Zamecnik[20] as described by Maxwell.[21] Fresh liver microsomes and pH 5 enzymes were prepared daily from two female Sprague-Dawley rats weighing 100–150 gm each. All quantities of fractions are expressed as gram equivalents (gm eq), i.e., the weight of liver from which the particular fraction was derived. Incubation mixtures routinely contained 0.15 *M* sucrose, 0.07 *M* KCl, 0.1 *M* tris buffer pH 7.3, 5×10^{-3} *M* $MgCl_2$, 5×10^{-3} *M* mercaptoethanol, 5×10^{-4} *M* ATP, 5×10^{-5} *M* GTP, 1×10^{-2} *M* potassium phosphoenolpyruvate, 100 μg crystalline pyruvate kinase (California Corp. for Biochemical Research), 5×10^{-5} *M* C^{14}-amino acid (1×10^6 cpm), 1×10^{-4} *M* each of 19 L-amino acids minus the C^{14}-amino acid, 0.2 gm eq microsome, 1.0 gm eq pH 5 fraction, and RNA as indicated. The final volume was 1.0 ml, and incubation was performed at 37° for 30 min. Protein was precipitated by the addition of 1 ml of 1 *N* perchloric acid, and the precipitate was washed according to the method of Hoagland *et al.*[16] The final precipitate was dissolved in 0.5 ml of 0.5 *N* NaOH, added to 10 ml of the scintillation solution described by Bray,[19] and assayed for C^{14} in a Packard liquid scintillation counter.

Results and Discussion.—In an attempt to characterize the pattern of response of RNA synthesis to steroid hormone administration, the influence of a single in-

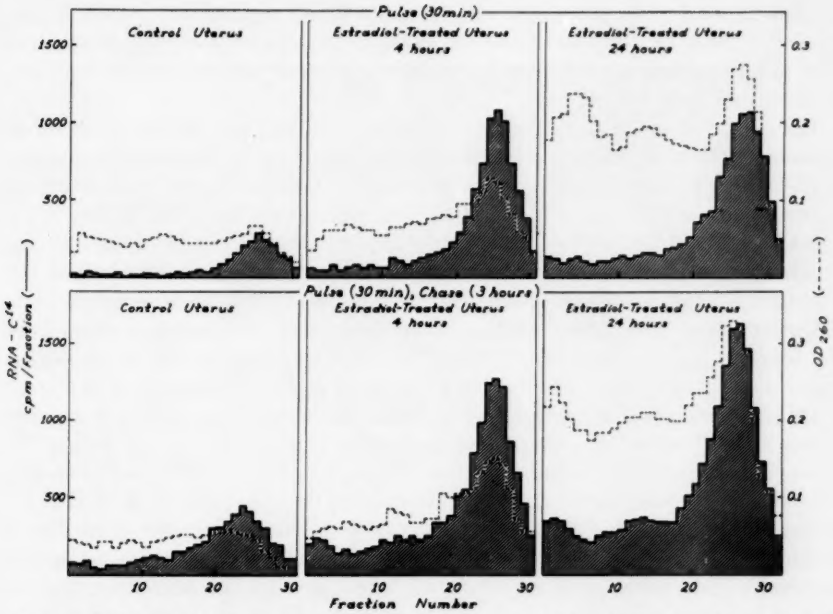

FIG. 1.—Sedimentation diagrams of uterine RNA from castrated (control) rats and from rats administered estradiol either 4 or 24 hr previously. The left hand part of each diagram represents the bottom of the tube and consequently the faster sedimenting RNA components. Each diagram represents the pooled tissue from seven rats.

travenous injection of 10 μg of estradiol-17β on the synthesis of the various fractions of RNA was studied in slices of uterus from castrated rats (Fig. 1). After a 30-min pulse with adenine-8-C^{14} (upper half) or a 30-min pulse with adenine-8-C^{14} followed by a three-hr chase with adenine (lower half), the RNA was separated by the phenol extraction technique of Perry[14] and centrifuged for 10 hr in a sucrose gradient density. Fractions were collected and analyzed both for radioactivity (the solid line) and for RNA concentration (the dotted line). In these diagrams, fraction 1 represents the bottom of the tube.

In the pulse experiment of the four-hr treated uteri (upper middle panel) there was a striking increase in the incorporation of adenine-C^{14} into the phenol extractable RNA; the radioactivity was localized in both the control and treated samples to a peak in the 4–6 S region corresponding to transfer RNA.[14] No radioactivity and only traces of RNA were detected in the regions which, according to Perry[14] correspond to ribosomal structural RNA (approximately 18 and 32 S). However, 24 hr after estradiol treatment (upper right panel) a major portion of the isolated RNA consists of these two peaks of ribosomal structural RNA.

The results of the studies of pulse labeling followed by a three-hr chase (lower half, Fig. 1) are similar to the pulse experiments; in each instance, the major peak of RNA-C^{14} is in the 4–6 S region. However, at the end of the $3^1/_2$-hr incubation, small amounts of radioactivity were found in the regions of structural RNA in the slices from the animals treated four hr previously (lower middle panel), and larger quantities of radioactivity were detectable in the regions in the uterine slices from

animals injected 24 hr previously with estradiol (lower right panel). Thus, it was concluded that the estradiol-induced increase in the synthesis of ribosomal structural RNA occurs as a relatively late event, several hr after demonstrable increases in the synthesis of 4–6 sRNA.

The probable explanation for this relatively late increase in the synthesis of ribosomal structural RNA can be derived from study of the histological changes of the uterine mucosa during estradiol treatment. From studies of RNA synthesis in tissue cultures of L strain fibroblasts, Perry has demonstrated that soluble RNA and a rapidly labeled labile RNA are synthesized in the chromatin of the nucleus; ribosomal structural RNA, on the other hand, appears to be synthesized in the nucleolus of the nucleus.[14] Moreover, it has previously been reported that, following testosterone administration to castrated rats, the shrunken nucleoli of the prostate undergo hypertrophy[22] and that the nucleolar volume of the uterus varies during the estrous cycle.[2] The histological studies demonstrated in Figure 2 were undertaken in an attempt to correlate the temporal sequence of the appearance of ribosomal structural RNA and the enlargement of the nucleoli of the uterus following estradiol administration. In both the hematoxylin and eosin and the azure-blue stained sections, it is quite evident that the nucleoli of uterine endometrium from the immature castrated rat are small and dense; the epithelium is cuboidal in nature, and the cytoplasm contains only trivial quantities of basophilic material. It is noteworthy that four hr following estradiol administration the nucleoli remain dense and pyknotic, and there is no evidence of transformation from the cuboidal to the columnar type of epithelium. By the end of the 24 hr period, however, striking changes have occurred. The nucleoli are large and pale, the epithelium has completed the transition to the columnar state, and the cytoplasm is laden with dense basophilic material. Thus, the enlargement of the endometrial nucleoli, the appearance of an RNA-rich cytoplasm, and the increase in the content of the ribosomal structural RNA all occur after marked increases in the synthesis of other RNA components. This demonstration is entirely consistent with the view that the synthesis of ribosomal structural RNA in uterus is a relatively late event following estradiol treatment. It should be noted that these results might be construed to be at variance with the study of Liao and Williams-Ashman,[13] who demonstrated that ribosomes (unsaturated with template RNA) continued to be present in rat prostate following castration. However, these investigators studied rats at a much shorter interval after castration (2–3 days), and it is possible that the cytoplasmic elements with slow turnover times had not had time to atrophy.

Next, an attempt was made to determine whether the RNA, synthesized within a four-hr period following estradiol treatment, included RNA with template activity (Table 1). Phenol-extractable uterine RNA from castrated and four hr estradiol-treated rats was assayed for template activity in the mammalian messenger RNA assay system as described by E. S. Maxwell;[21] this system consists of rat liver microsomes and pH 5 enzyme, ATP, GTP, an ATP generating system, and a mixture of amino acids. As shown in Table 1 the addition of RNA from estradiol-treated uterus enhanced amino acid incorporation into protein about 50 per cent when lysine-C^{14} was used as the tracer and about 75 per cent using histidine-C^{14}, whereas equal amounts of uterine RNA from the castrated control had no effect on amino acid incorporation.

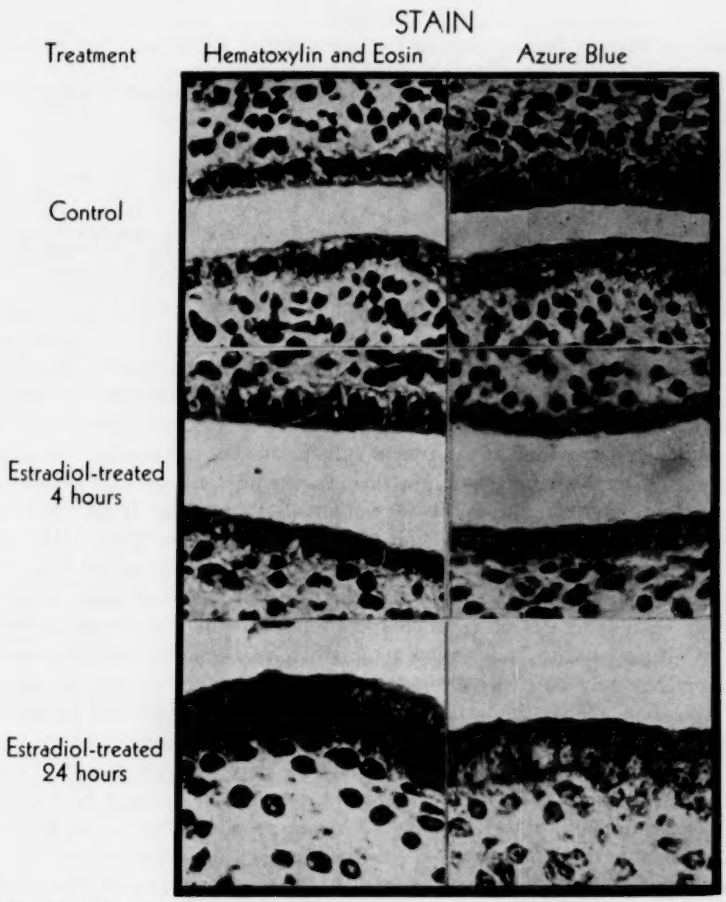

FIG. 2.—Photomicrographs of the uterine mucosa from castrated (control) rats and from rats administered estradiol either 4 or 24 hr previously. In each instance the magnification is 800 ×.

The influence of four additional preparations of phenol-extracted RNA from control and four-hr estradiol-treated uterus on amino acid-C^{14} incorporation by rat liver enzymes is illustrated in Table 2. Using three different C^{14}-labeled amino

TABLE 1

INFLUENCE OF UTERINE RNA ON AMINO ACID INCORPORATION BY RAT LIVER ENZYMES

System	C^{14} incorporation into protein from: L-Lysine-U-C^{14} (cpm)	L-Histidine-U-C^{14} (cpm)
Complete system	92	76
Complete system plus RNA_E	136	130
Complete system plus RNA_C	96	66
Complete system minus microsomes	12	0
Complete system minus pH 5 enzyme	34	6
Complete system minus ATP, GTP, and pyruvate kinase	4	22

Reaction mixture is described in *Methods*. The quantities of RNA_E and RNA_C were 1.1 and 1.4 OD_{260} units, respectively.

TABLE 2

INFLUENCE OF VARIOUS PREPARATIONS OF UTERINE RNA ON AMINO ACID INCORPORATION BY RAT LIVER ENZYMES

Exp. no.	C^{14}-amino acid	Per cent enhancement by added RNA	
		RNA_E	RNA_C
1	Lysine	20	11
	Histidine	18	1
2	Leucine	37	19
3	Leucine	20	0
4	Lysine	31	17

Reaction mixture is described in *Methods*. The quantities of added RNA_E and RNA_C varied from 0.9 to 1.3 OD_{260} units: in each instance, the results have been expressed as per cent enhancement per 1.0 OD_{260} unit.

acids in the assay system, the enhancement by the RNA_E varied from 18–37 per cent, whereas the addition of the control RNA produced 0–19 per cent enhancement. Although the ability to enhance protein synthesis did vary among preparations, it should be emphasized that in every instance the enhancement was at least twice as great in the four-hr estradiol-treated tissues as in the control preparation. While these results are consistent, the degree of enhancement is not nearly so large as has been reported by Nirenberg and Matthaei for synthetic polyribonucleotides[23] or by Barondes, Dingman, and Sporn for mammalian messenger RNA[24] in bacterial assay systems; the results reported here (18–73 per cent enhancement/OD_{260} RNA_E) are, however, comparable to those reported for rat liver template RNA in rat liver enzymes by Maxwell.[21] The difference between the bacterial assay system and the mammalian assay system is probably twofold; the mammalian assay system is partially saturated with endogenous template RNA, and the naturally occurring template RNA may be either altered or partially damaged during the extraction and purification. That the latter is a likely possibility is suggested by the recent demonstration by Singer, Jones, and Nirenberg that the template activity depends, in part, on the ordered secondary structure of the polyribonucleotide.[25] Thus, the assay of the template activity of these RNA preparations cannot be regarded as absolute measurements of the template RNA content of the intact tissue in question, and it is possible that the magnitude of the increased template activity of uterine RNA within four hr following estradiol treatment is much greater than has been demonstrated in these experiments.

Studies which further characterize the template RNA activity of rat uterus are shown in Figure 3. When the various RNA fractions obtained following pulse labeling with adenine-C^{14} and gradient density centrifugation were assayed for their ability to enhance amino acid incorporation (the dotted lines), all the template activity was found plastered to the bottom of the tube (here indicated as tube 0), indicating that in this tissue, at least, messenger RNA must be greater than 32 S in size. This observation confirms the report by Barondes, Dingman, and Sporn that the template RNA of rat liver nuclei is at least 40 S in size.[24]

As the result of these studies, three general conclusions may be drawn. First, a direct demonstration has been made that uterine RNA with template activity, as assayed in an amino acid incorporating system from rat liver, is increased within a four-hr period following estradiol administration to castrated rats. Second, although the synthesis of all RNA components does increase following estradiol administration, the synthesis of RNA in the nuclear chromatin appears to increase first, resulting in an increase both in template RNA and in transfer RNA; the

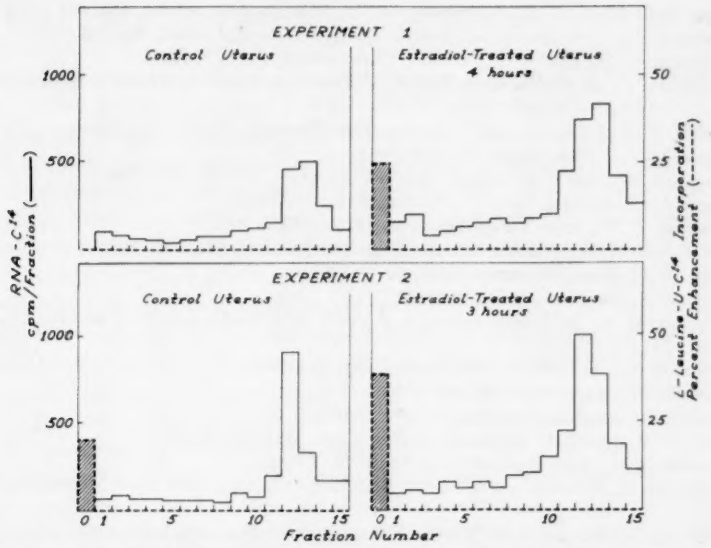

FIG. 3.—Sedimentation diagrams of template activity of uterine RNA from castrated (control) rats and from rats administered estradiol either 3 or 4 hr previously. Each diagram represents the pooled tissue from 10 rats. The extractions and centrifugations were performed following a 30 min pulse labeling of uterine slices with adenine-8-C^{14}. In each instance radioactivity was obtained in tube 0; however, due to the method of collection it is impossible to prevent contamination of the bottom of the tube with radioactivity from the last samples collected. As a consequence, no radioactive data were reported for these tubes.

enhancement of the synthesis of ribosomal structural RNA, on the other hand, appears to be a somewhat later event. Finally, since transfer RNA is not rate-limiting for protein synthesis in accessory sex tissue,[9-11] it seems justifiable to conclude that the early acceleration of protein synthesis in this tissue by estradiol administration is secondary to an increase in template RNA.

Summary.—On the basis of histological and chemical studies it is concluded that following estradiol administration chromosomal RNA synthesis is enhanced, as the result of which transfer RNA and template RNA activities are increased. The subsequent enhancement of the nucleolar RNA—the structural RNA of the ribosomes—appears to be a relatively late event. Finally, since the enhancement of RNA synthesis in the nuclear chromatin is an early result of estradiol action, it is likely that the syntheses of other RNA components and of protein are subsequent responses.

The author is indebted to Dr. Rolland Reynolds for assistance in the histological studies and to Joanne Sherwood for able technical assistance.

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† Established investigator of the American Heart Association.

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*COUPLED PHOTOREDUCTION OF UBIQUINONE AND PHOTOOXIDATION OF FERROCYTOCHROME C CATALYZED BY CHROMATOPHORES OF RHODOSPIRILLUM RUBRUM**, †

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Communicated by Martin D. Kamen, May 27, 1963

A light-induced reaction between bacteriochlorophyll and a cytochrome of type *c* in *Chromatium* cells has been reported by Chance and Nishimura,[1] who showed a temperature-independent oxidation (down to 80°K) of the cytochrome under anaerobic conditions at a rate reported to be 0.39 μM Fe^{++}/sec. Duysens[2] had earlier described a light-induced oxidation of cytochrome in *Rhodospirillum rubrum* under anaerobic conditions. In the experiments of Duysens, as well as in those of Chance and Nishimura, cytochrome was again reduced in the dark. Clayton[3] showed that the light-induced oxidation of cytochrome in *Chromatium* chromatophores is closely connected to the absorption changes of a special component of bacteriochlorophyll. Evidence to date therefore favors a cytochrome as the electron donor reacting directly with bacteriochlorophyll upon illumination.

Ubiquinone (UQ)† has been implicated as a primary electron acceptor in bacterial photosynthesis by Clayton,[4] who related the light-induced absorption changes in the 240–350 mμ range to the reduction of UQ in chromatophores from *Chromatium*, *R. rubrum*, and *Rps. spheroides*. He also showed there was almost a 1:1 relation-

ship between the cytochrome oxidized and the UQ reduced in the light reaction, if the absorbancy change at 280 mμ could be attributed to the quinone. Bales and Vernon[5] have correlated the rapid photooxidation of reduced 2,6-dichlorophenolindophenol by chromatophores of *R. rubrum* with absorption changes at 280 mμ, implicating UQ as the electron acceptor in this reaction.

Vernon and Kamen[6] reported a photooxidation of added ferrocytochrome *c* by *R. rubrum* chromatophores in the presence of oxygen. Vernon[7] later demonstrated that this reaction could take place under strict anaerobicity upon the addition of fumarate or NAD. Smith[8] concluded that the photooxidation of added cytochrome *c* was not enzymic since chromatophores which had been heated at 70° for 30 min still exhibited the reaction, even though proteins of the extract were precipitated.

Lindstrom[9] studied the photooxidase activity of chromatophores using reduced 2,6-dichlorophenolindophenol as the electron donor, and concluded that such photooxidations are valid measurements for the oxidant in bacterial photosynthesis.

The experiments reported here show a very rapid photooxidation of added mammalian cytochrome *c* by chromatophores of *R. rubrum* with added UQ as the electron acceptor. Reduced UQ reduces the oxidized cytochrome *c* both chemically and enzymically in the dark, making the system reversible.

Methods and Materials.—*R. rubrum* cells were grown in a medium containing malate, glutamate, acetate, and ammonium chloride, as described previously.[10] Chromatophores were prepared by 2 min sonic oscillation (Raytheon, 10 kc) of the whole cells, followed with two washings by centrifugation of the particles sedimenting from 20,000–100,000 × *g*.[10]

Reactions were followed in a modified Beckman DB spectrophotometer. A cover for the cell compartment was constructed having a square opening, 14 mm on a side, directly above the sample cell. A square tube attached to the under side of the cover funneled the light directly to the sample, thereby eliminating stray light from reaching the phototube. In addition, cuvettes containing 1 F $CuSO_4$ were placed between the sample (and reference) and the phototube to minimize any effect of scattered light. The light source was an American Optical Universal microscope lamp placed 12 cm above and focused on the 1 ml sample. The lamp used a 6.5 v, 2.75 amp clear glass bulb and the variable voltage selector was set at 7.5 v, except where otherwise stated. The light beam passed through 2 cm of water and a Corning glass filter No. 2403 before passing through the opening in the cover of the cell compartment. Chlorophyll was determined by the method of van Niel and Arnold.[11] Reduced quinone was prepared according to the procedure of Green and Burkhard.[12] Horse heart cytochrome *c* obtained from Sigma Chemical Co. was used in these experiments. The experiments reported here were carried out aerobically, since the degree of anaerobicity had no effect on the rate of the reaction. The rate of oxygen-dependent cytochrome *c* photooxidation by *R. rubrum* chromatophores, which was reported by Vernon and Kamen,[6] is much slower than the quinone coupled photooxidation.

Results.—Figure 1 shows the reduction of cytochrome *c* which occurred when reduced UQ_2 was mixed with cytochrome *c* and *R. rubrum* chromatophores. Subsequent illumination produced an immediate oxidation of the cytochrome. The sequence could then be repeated by turning off the light, waiting until the cytochrome was completely reduced, and again illuminating. After the reaction had

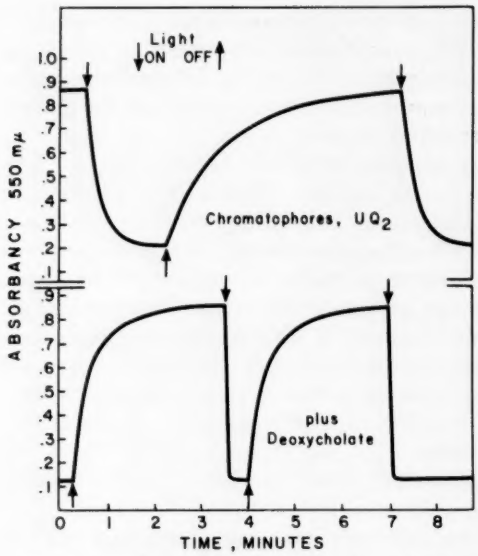

FIG. 1.—Photooxidation of cytochrome *c* by chromatophores of *R. rubrum* in the presence of ubiquinone. The reaction mixture consisted of 0.47 mmoles sucrose, 0.03 mmoles phosphate buffer (final pH 7.0), 9.0 μmoles KCl, 36 mμmoles cytochrome *c*, 110 mμmoles reduced UQ_2 added in 0.01 ml ethanol,and 0.01 ml *R. rubrum* chromatophore preparation containing 0.011 mg bacteriochlorophyll in a final volume of 1.0 ml. The reaction was followed at 550 mμ in a modified Beckman DB spectrophotometer with external recorder. The cell compartment was maintained at 25°C with circulating water. At the points indicated in the upper curve, the light was turned on, resulting in an oxidation of the cytochrome *c* which had previously been reduced by the reduced UQ_2. The lower curve shows the effect of adding 0.02 ml of a 10% solution of sodium deoxycholate to the above reaction mixture. The initial portion of this curve shows the reduction of cytochrome *c* by reduced UQ_2 in the dark. These experiments were performed under aerobic conditions. However, as discussed in the *Methods* section, the degree of anaerobicity did not affect the rate of cytochrome photooxidation.

been allowed to complete two cycles, the reaction mixture (1 ml) was made 0.2 per cent in deoxycholate by addition of 0.002 ml of a 10 per cent solution. Presence of the bile salt increased the rates of both the dark and light reactions. From other experiments it appeared that the detergent acted not only by increasing the solubility of UQ, but also by making the reaction site on the chromatophore more available to the reactants. As expected, the effect of deoxycholate was more pronounced when UQ_6 and UQ_{10} were used, since in the absence of the bile salt both the dark and light reactions were comparatively slow.

The rates of the photoreactions in deoxycholate-treated chromatophores, using UQ_6 or UQ_2, generally ranged from 6–8 mmoles cytochrome *c* oxidized/hr/mg chlorophyll, with one series of reactions averaging 9 mmoles/hr/mg chlorophyll. This rate exceeds by more than fivefold, on an equivalent electron basis, the value obtained by Vernon[13] for the photooxidation of reduced 2, 6-dichlorophenolindophenol in the presence of NAD (0.553 mmole/hr/mg chlorophyll).

Figure 2 shows the dependence of the photooxidation of reduced cytochrome *c* upon the presence of UQ_6. The first curve in this figure shows that chromatophores, in the presence of cytochrome *c* (a small amount of which is already in the reduced form) and 0.2 per cent deoxycholate, are capable of rapidly photooxidizing a small amount of cytochrome in the absence of added UQ. The amount of cytochrome oxidized is about 3.8 mμmoles/ml. If this corresponds to endogenous quinone reduced, the photoreaction results in the reduction of 1.9 mμmoles/ml of UQ_6. Since there were 11.4 mμmoles/ml of bacteriochlorophyll in the reaction mixture, the ratio of chlorophyll to quinone would be about 6 to 1 in this preparation of *R. rubrum* chromatophores. There is at present no report in the literature which compares the amount of UQ in *R. rubrum* chromatophores directly with chlorophyll

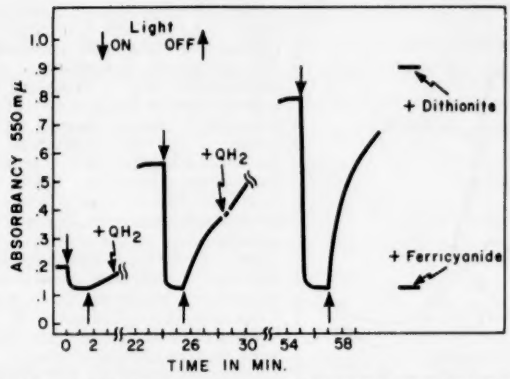

FIG. 2.—Dependence of cytochrome *c* photooxidation on added UQ. The reaction mixture contained 0.47 mmoles sucrose, 9.0 μmoles KCl, 0.03 mmoles phosphate buffer (final pH 7.0), 41 mμmoles cytochrome *c*, 0.02 ml of 10% sodium deoxycholate solution, and 0.01 ml *R. rubrum* chromatophores containing 0.011 mg bacteriochlorophyll in a volume of 1.0 ml. At places indicated, 11 mμmoles of reduced UQ_6 were added in 0.001 ml ethanol. The reaction was followed at 550 mμ as indicated in Fig. 1. See text for explanations.

content. Vernon[13] used the results of three separate investigations on content of UQ,[14, 15] cytochrome, and chlorophyll[16] to arrive at an approximate value of 3 chlorophylls to 1 UQ for *R. rubrum*. The first addition of 11 mμmoles of reduced UQ resulted in a rather slow reduction of cytochrome *c* which required about 18 min to reach completion. The sample was then illuminated (center curve of Fig. 2), resulting in the oxidation of 20 mμmoles of cytochrome *c* as determined by the change in absorbancy at 550 mμ, using a millimolar absorbancy index of 21.0.[17] After turning off the light and allowing the dark reduction of cytochrome *c* to proceed for a few minutes, a second aliquot of reduced UQ_6 was added (11 mμmoles). A greater quantity of cytochrome then became reduced, which was completely oxidized upon illumination (third curve).

The apparent discrepancy in the ratio of cytochrome photooxidized to quinone added, shown in the third curve of Figure 2 (31:22), compared to that shown by the center curve (20:11), is most likely due to oxidation of the reduced quinone by oxygen in the air. This was indicated by other experiments in which limiting amounts of UQ were added. The amount of cytochrome *c* photooxidized gradually decreased through several cycles. With an excess of reduced quinone, however, the light-dark cycle could be repeated many times during a 2–3 hr period without appreciable change in the light-dependent reaction. The dark reaction tended to become slower, however, because of the decrease in reduced UQ-cytochrome *c* reductase activity. It can be noted by the absorbancy reading in the presence of ferricyanide (cytochrome *c* completely oxidized) that the light-induced reaction with excess quinone resulted in complete oxidation of the added cytochrome.

Evidence that the added quinone was reduced during the photooxidation of cytochrome was obtained from measurements in the 260–290 mμ region. Figure 3 shows the absorption spectra of oxidized UQ_2 obtained in the dark (curve *A*) and reduced UQ_2 produced in the light (curve *B*). Almost identical curves were obtained upon repeating the light-dark sequence on the same sample.

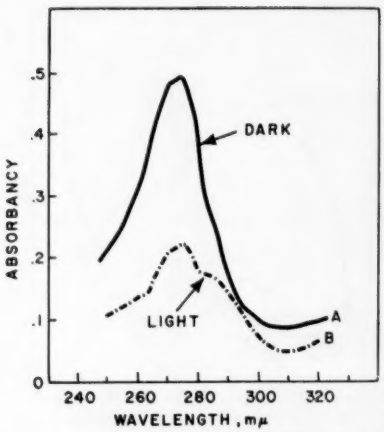

FIG. 3.—Spectral evidence for UQ photoreduction. A 6 ml reaction mixture was made up containing the same final concentrations of sucrose, KCl, phosphate, cytochrome *c*, and deoxycholate as mentioned in Fig. 2. The final concentration of *R. rubrum* chromatophores corresponded to a bacteriochlorophyll concentration of 0.012 mg/ml. A 1 ml sample of this reaction mixture was withdrawn and placed in a cuvette in the sample compartment of the spectrophotometer. The remainder of the mixture was divided equally between two cuvettes to be used as references. One reference cell contained cytochrome *c* in the oxidized form while the cytochrome in the other reference cell was reduced with $NaBH_4$. To the sample cell was added 22 mμmoles of reduced coenzyme Q_2 in 0.002 ml of ethanol. After waiting a few minutes for the cytochrome *c* to become reduced, the light was turned on. With the light on (cytochrome *c* oxidized, quinone reduced) a difference spectrum was taken against the reference containing oxidized cytochrome *c* (curve *B*). A check at 550 mμ showed that the degree of oxidation in the reference cell was essentially the same as in the illuminated sample cell. The light was then turned off and, after reduction of the cytochrome by the reduced quinone, a difference spectrum was taken against the reference cell containing reduced cytochrome *c* (curve *A*). A second illumination (again using the reference cell containing oxidized cytochrome *c*) resulted in a difference spectrum which was the same as curve *B*. For these experiments it was necessary to filter the incident light (variable voltage selector set at 4.0 volts; see *Methods* section) through two Corning glass filters (numbers 2403 and 2550) and 2 cm of water, and also to use 1 cm of water as a barrier filter between the illuminated sample and phototube to minimize any effect of scattered light on the spectra taken in the UV region during illumination.

Curve *B* shows the presence of some oxidized quinone in the light. Although cytochrome and quinone were added in approximately equivalent amounts (2 molecules of cytochrome to 1 molecule of quinone), it is apparent that in this experiment not all the quinone was originally in the reduced form. However, the curves shown were taken within a few minutes of each other, and definitely show the changes in redox state of quinone which accompany cytochrome *c* photooxidation.

In agreement with previous observations on the heat stability of the photooxidation reactions of *R. rubrum* chromatophores [8, 9] it was found that good activity remained in chromatophores which had been incubated at 60° for 3 hr. However, when heated to 90° for 2 min no activity could be observed even after sonication of the heated particles.

Discussion.—The rapid photoreaction reported here, involving the oxidation of added cytochrome *c* and a simultaneous reduction of added UQ by *R. rubrum* chromatophores, is a model system for the primary events currently believed to take place when whole cells are illuminated. Investigators have shown that photooxidation of a *c* type cytochrome occurs very early in the sequence of changes caused by illumination of cells as well as chromatophores of photosynthetic bacteria.[1, 18–22] Quinone has been implicated as an electron acceptor in the photooxidation of reduced 2,6-dichlorophenolindophenol,[5] and Clayton[4] has proposed that UQ acts as a primary electron acceptor in bacterial photosynthesis. The demonstration that chromatophores are capable of the rapid photoreaction described in this paper lends support to the current concept of cytochrome and quinone function in the primary photochemical event.

The ability of chromatophores to carry out the reduction of a quinone by the same chlorophyll system which oxidizes a cytochrome may well represent one of the

fundamental differences between the bacterial system and that of higher plants. The current evidence indicates that quinone in the plant system is reduced by a different chlorophyll system than that which oxidizes the cytochrome.[23] This could account for the observation that *R. rubrum* chromatophores demonstrate a true cyclic photophosphorylation, while chloroplasts do not perform this function at an appreciable rate, unless phenazine methosulfate is added to serve as a cofactor. Attempts thus far to show a rapid photooxidation of cytochrome *c* and a coupled reduction of UQ or plastoquinone in chloroplasts or chloroplast fragments under a variety of conditions have not been successful, further demonstrating a basic difference between the two photosynthetic systems.

The observation of reduced UQ-cytochrome *c* reductase activity in *R. rubrum* chromatophores, which experiments now in progress show is antimycin-sensitive, furnishes evidence of a dark electron transfer system similar to that of mitochondria. If a comparison can be made between the sites of phosphorylation in the two systems, ATP formation would be expected to occur during the transfer of electrons from the reduced quinone to the *c* type cytochrome in bacteria. The photooxidation by *R. rubrum* chromatophores of added cytochrome *c* with the simultaneous photoreduction of added UQ and the subsequent re-reduction of cytochrome *c* in a dark, antimycin-sensitive reaction may very well represent the flow of electrons which results in cyclic photophosphorylation.

The author wishes to thank Dr. L. P. Vernon for his comments and suggestions, and Ann Tirpack for her assistance during the course of the investigation. UQ_2 was a gift of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

* Contribution No. 117 of the Charles F. Kettering Research Laboratory.

† The term "chromatophore" designates the photosynthetically active particle or fragment which is obtained upon rupture of the intact cell by sonic oscillation. The name Ubiquinone (UQ) is used as suggested by the Commission on Enzymes of the International Union of Biochemistry.[24]

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*EVIDENCE FOR MESSAGE READING FROM A UNIQUE STRAND OF PNEUMOCOCCAL DNA**,*†

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DNA banded in CsCl gradients at pH 12 has an increased buoyant density due to binding Cs^+ ions to guanine (G) and thymine (T) residues, which ionize when the DNA denatures near pH 11.[1] In the course of other experiments we observed that pneumococcal DNA had an unusually broad and rather flat-topped profile under these conditions, as compared to its denatured band at neutral pH (Fig. 1).

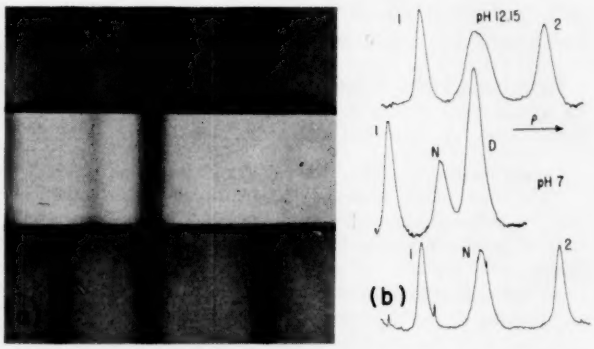

FIG. 1.—Ultraviolet photographs and densitometer tracings of typical pneumococcal DNA bands in CsCl, with reference bands of dAT polymer (1) and *M. lysodeikticus* DNA (2). *Top:* pH 12.15; *middle:* dAT plus native and heat-denatured pneumococcal DNA, pH 7; *bottom:* native, pH 7. Centrifuged 21 hr at 44,770 rpm, 25.0°C. The dAT and *M. lysodeikticus* bands at pH 12 are still rather sharp, whereas the pneumococcal band suggests overlapping peaks. The widths at half maximum are in the ratio 1:1.2:1.9 for native, denatured at pH 7, and pH 12, respectively. Tracings were drawn directly on the figure by a Joyce-Loebl microdensitometer.

Although the mole fraction of G + T equals 0.50 in double-stranded DNA, they need not be equally distributed between the complements. Vinograd *et al.*[1] looked for evidence of strand fractionation in *E. coli*, *M. lysodeikticus*, and T-4 phage DNAs, but found none. Our results generally agree with theirs, in that the effect is certainly small in most species. We have, however, studied the pneumococcal case further, and the results appear to be of significance regarding strand separation,[2] transformation by denatured DNA,[3, 4] and message transcription.

Pneumococcal-transforming DNA carrying four drug resistance markers (streptomycin—S; erythromycin—E; bryamycin—B; and novobiocin—N) was banded in CsCl (A. D. McKay, Inc.) in the SW-39 rotor of the preparative ultracentrifuge at 35,000 rpm for 4 days, as described previously.[3, 5] The pH was adjusted by addition of NH_4OH and NaOH to a value of pH 12.5 ± 0.3, as read on a radiometer TTT-1 pH meter. Bands were fractionated by puncturing the tube and collecting 0.008 ml drops, from one to three per fraction in various experiments.

Transformation assays, P^{32} label, or absorbance measurements served to locate the DNA peaks.[6]

Single fractions were then rebanded in the analytical centrifuge at pH 12 and at pH 8 in CsCl (Maywood Corp., optical grade), with added references of dAT polymer (gift from C. Schildkraut) and *M. lysodeikticus* DNA. Figure 2 shows typical results where such fractions differ by 0.005_1 g/ml at pH 12.1 and only 0.001_2 at pH 8.6.[7] At both pH's, the two fractions straddle the density of unfractionated pneumococcal DNA. The broad band at pH 12 is clearly due to real density heterogeneity which is largely reversed on return to neutral pH. Variation of pH from 11.7–12.3 does not affect the band spread. On return to pH 7–8, however, if the concentration per drop is high, substantial spontaneous renaturation occurs.[1] The fractions must be heated in order to see the densities shown in Figure 2*b*. This does not occur to the same extent at lower concentrations of DNA in the preparative band.

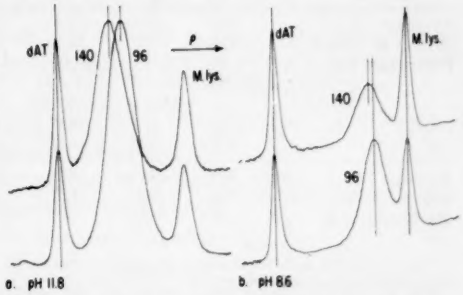

FIG. 2.—Rebandings in the analytical centrifuge of portions of single fractions of pneumococcal DNA from a preparative CsCl gradient at pH 12.2 (0.7 mg DNA, 115 hr at 35,000 rpm, 25°C). The peak was near fraction 117: (*a*) rebanded at pH 11.8, $\Delta\rho = 0.005_1$ g/ml; (*b*) rebanded at pH 8.6 after heating at 100°Cfor 10 min, $\Delta\rho = 0.001_2$ g/ml. Same references and conditions as in Fig. 1.

In assaying the fractions for transforming activity, we also tested the effect of varying the time allowed for phenotypic expression of the drug resistance. (Details of the assay have been described previously.[3, 5]) Depending on the properties of the drug and on factors still poorly understood, but perhaps related to dominance versus recessiveness of the gene transferred, it normally takes anywhere from a few minutes to 80–90 min for a drug resistance phenotype to be expressed. S and E require the longer time, and B and N a much shorter time. If there were a difference in the ability of the presumed separated strands to be read, then the "expression curve" might be affected. Figure 3 shows the result for novobiocin resistance transformation when the transformed cells were challenged by plating on the drug at 20 min and 90 min after DNA addition. The peak of activity moved several fractions (0.002–0.003 g/ml) toward higher density as more time was allowed for phenotype to develop. Five experiments each showed this phenomenon for N at pH 12. No such shift was observed in denatured DNA banded at pH 7, nor did bryamycin resistance show a shift at either pH. The expression of S and E is sufficiently slow that the 20 min challenge does not give reliable numbers, and we are not yet certain whether they move significantly.

Since N showed the largest effect, further experiments were concentrated on it, and opposite fractions were selected so as to give nearly equal concentrations of final activity. This meant that S in particular was very unequally distributed between fractions, because S is concentrated in the high density side of the peak, as it is in native DNA at pH 7 also.[5]

TABLE 1

RENATURATION OF TRANSFORMING ACTIVITY IN FRACTIONS FROM pH 12 CsCl GRADIENT*

Fraction	Before† S	Before† N	Before† E	Before† B	After S	After N	After E	After B
	Dilution—10 ×				Dilution—50 ×			
96	359	268	288	212	798	455	930	816
140	158	196	140	138	179	249	244	343
Average	258	232	214	175	489	352	587	579
96 + 140 Mixed	233	190	221	179	692	528	587	434
Ratio mix/avg.	0.90	0.82	1.03	1.02	*1.41*	*1.50*	1.00	0.75
	(0.60)†	(1.20)	(0.82)	(1.35)	(*1.35*)	(*1.49*)	(0.98)	(0.85)
	Dilution—100 ×				Dilution—200 ×			
106	542	375	590	477	426	260	472	334
126	187	330	250	249	194	262	230	194
Average	364	352	420	363	310	261	351	264
106 + 126 Mixed	362	360	425	361	321	320	347	278
Ratio mix/avg.	1.00	1.02	1.01	1.00	1.03	*1.23*	0.99	1.05
					(1.03)	(*1.18*)	(0.92)	(0.91)

* DNA samples were renatured as described in text. The data are the numbers of colonies resistant to each drug, observed from parallel platings of the indicated total dilutions of cultures transformed by the DNA fractions.

† 96 and 140 were heated before this assay. 106 and 126 were not, and showed substantial spontaneous renaturation already.

‡ Ratios in parentheses are from results of fourfold further plating dilutions of the same cultures, and are less reliable statistically. The expected standard error from Poisson statistics is 3–8% for the individual counts cited above, and this is about the reliability we find for such data (cf. ref. 5).

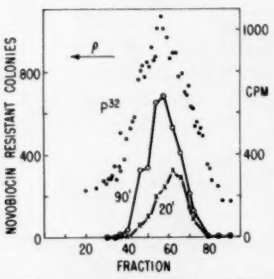

FIG. 3.—Distribution of P^{32}-labeled pneumococcal DNA and of novobiocin resistance phenotype in cells transformed with these DNAs in fractions from a preparative CsCl gradient at pH 12.2 (0.05 mg DNA, 118 hr at 35,000 rpm, 25°C). Cultures were transformed at time 0 with 1/50 of the DNA per fraction, and plated on drug-containing agar at 20 min and again at 90 min. DNAase was added at 10 min to destroy remaining extracellular DNA.

Figure 4 and Table 1 give the results of renaturation tests.[8] 0.2–0.3 ml vol of the DNAs, in approximately 0.5 molal CsCl plus 0.35 *M* NaCl, 0.035 *M* Na citrate (2× "SSC"), were sealed in tubes and held at 65.5 ± 0.5° for 4 hr., with slow shaking, and allowed to cool slowly to room temperature over a further 4–6 hr period. Total DNA was 10–15 micrograms/ml in these experiments, and both it and N activity were nearly equal in the various pairs of tubes. With the long period of heating it was hoped to minimize differences due to *rate* of renaturation and to approach a final state more independent of exact concentration

After renaturation the densities of all the samples had shifted back toward that of native DNA, but the bands of renatured mixtures were consistently sharpest (see Fig. 4). These fractions were not pure, but only enriched in their heavy or light components. Also, the dense leading edge separated better than the light trailing edge, which was subject to more mixing during drop collection. Most important, however, *the single fractions each showed a substantial trail of material at the position of fully denatured DNA, which was greatly reduced in mixtures of these fractions renatured simultaneously.* Complementary components existed therefore in the separate fractions.

Two fractions nearer the center of the same preparative band (106 and 126) showed quantitatively less but qualitatively similar complementarity by the rebanding test, and Table 1 summarizes transformation assays on these sets of

fractions. Before renaturation and *after* for B and E markers, the mixtures gave numbers of transformants essentially identical to the average of the individual fractions, whereas N showed significantly higher activity in the mixtures. The relative concentrations of S in the fractions differed sufficiently that we hesitate to draw firm conclusions about its behavior. The mixture of fractions 106 and 126 showed less renaturation here also. Other data confirm this trend (e.g., Fig. 6).

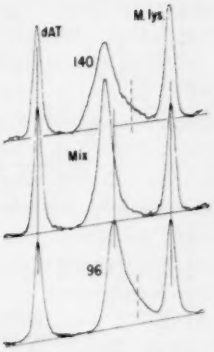

FIG. 4.—Bands of fractions 96 and 140 (cf. Fig. 2) after renaturation separately and in a mixture (see text). Centrifuged simultaneously in CsCl, pH 8.1, in 4 cell F-rotor, 20 hr, 44,770 rpm, 25.0°C. The quantities of DNA put in were the same, and the areas under the peaks are the same within 7%. The dotted lines mark the centers of the respective peaks before renaturation (Fig. 2*b*).

Returning to the variation of expression time for novobiocin resistance (Fig. 3), we note that the heavy fractions increased in activity between 20 and 90 min, much more than did the light fractions. Complete expression versus time curves were carried out as follows. Two ml samples of competent recipient cells were transformed with appropriate concentrations of two DNA fractions at time 0, an excess of DNAase added at 5 or 10 min, and samples withdrawn and plated at varying times up to 90 min. Thirty second intervals were allowed for manipulations, and experiments were done in both sequences in order to eliminate spurious time delay effects. More commonly, the samples were plated in an order such that timing errors would minimize the effect.

Independent of order, all experiments gave the same result (Figs. 5 and 6). Novobiocin resistance was usually fully expressed in 45 min and the increase from there on was due to multiplication of transformed cells in the liquid culture. In some experiments the cells appeared to be synchronized[9] and this altered the kinetics of expression somewhat. Before 45 min, however, the light fractions expressed much more rapidly than did the heavy. When normalized to equal final activity, the ratio of colonies observed from light and heavy DNAs decreased with time by a factor of 2.5–4.3 (depending on proximity to center) to near 1.0 at 45 min and later times. Some of the early expressed activity in heavy DNAs was presumably due to contamination with light DNA.

Inasmuch as there was a possibility that variations in degree of secondary structure could account for the differences in density and expression time (though probably not for differences in renaturation and in behavior of different markers), controls were run on denatured versus native unfractionated DNA and on pairs of fractions, (*a*) as collected, (*b*) after heating to remove spontaneous renaturation, and (*c*) after renaturation with wild-type DNA (Fig. 6*b*). Denatured, renatured, and native DNAs expressed almost identically (with a possible small trend toward more rapid expression of native), and the difference between heavy and light fractions was maintained through all denaturation and

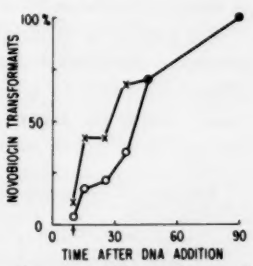

FIG. 5.—Expression of novobiocin resistance phenotype in cultures transformed by fractions 96 (O—O) and 140 (×—×) (cf. Fig. 2).

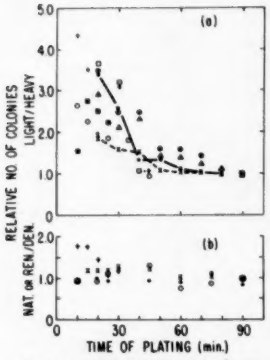

FIG. 6.—(*a*) Ratio plot showing increased early expression of novobiocin resistant transformants from light pH 12 fractions compared to those from heavy fractions, tested in pairs and normalized to equal activity at 90 min expression. ●—● fr. 120/fr. 90, ×---× fr. 116/fr. 96, tested simultaneously, from same preparative band where N peak was at fr. 104. The effect is less for the inner pair. Other points are from separate experiments. (*b*) Same plot for native compared to heat-denatured DNA (+, ×); and for a light pH 12 fraction (○) and a heavy one (●), renatured with wild-type DNA and compared in each case to the mixture before renaturation.

renaturation conditions.[10] Native DNA expressed about as the average of light and heavy fractions (Table 2).

Discussion.—Our observations to date include: (*a*) pneumococcal DNA banded at pH 12 shows a round-topped band suggestive of two overlapping distributions, both of which are presumed to be internally heterogeneous; (*b*) examination of fractions from such a band shows that they differ substantially in density at pH 12 and much less so at pH 8; (*c*) renaturation is more extensive and complete in mixtures of opposite fractions than in a single fraction alone, by both banding and transformation tests; (*d*) the peak of novobiocin resistance activity moves toward higher density as more time is allowed for phenotypic expression; (*e*) expression time tests confirm that light fractions express their activity more rapidly than dense fractions, and show that this effect is completed after 45 min expression time; (*f*) the expression of the light fractions is more rapid than that of either native or denatured unfractionated DNAs, which express at nearly the same rate.

We interpret these observations to mean that a significant portion of complementary strands of pneumococcal DNA differ sufficiently in their guanine plus thymine content to be separated by at least 0.005 g/ml in a CsCl density gradient at pH 12. At neutral pH they differ a small amount in some aspect of their composition, the details of which await further study. Individual sections of the DNA may differ more or less, and apparently the B-and E-containing molecules are not asymmetric in this respect. Vinograd *et al.*[1] measured 0.045 g/ml as the average increment in density for the total DNA, of 50% G + T. Tentatively scaling from this figure, 0.005 g/ml would correspond to a 5–6% difference in G + T, or perhaps 47 and 53% in the respective average strands of fractions 96 and 140 (e.g., Fig. 2).

The further interpretation, then, is that the light fraction (low G + T) contains a strand of novobiocin resistance marked DNA which can be read into phenotype almost immediately on uptake by the cell, whereas the dense strand requires more time. Since the latter strand catches up in about 45 min, which is only slightly

TABLE 2

RELATIVE EXPRESSION RATE WITH LIGHT, HEAVY, AND UNFRACTIONATED NATIVE DNAs*

Time (min)	Fr. 89	Fr. 121	Native	Avg. 89, 121
10	4.3	6.7	5.1	5.5
15	4.6	12.6	10.0	8.6
20	10.0	25.2	17.6	17.6
25	15.7	35.3	23.2	25.5
30	...	38	28	...

* As per cent of 95 min novobiocin-resistant colonies. Fractions from pH 12.5 CsCl gradient, peak N activity at fr. 104.

longer than one generation time of the cells (40 min in these conditions), it is evident that this strand must be replicated before it can be read. This says then that effective messenger RNA is copied from only one of the strands of DNA, either of which can transform the cell's genotype.

It is of interest to relate the above results to other work on transformation. Fox[11] found an eclipse period of a few min following addition of DNA to recipient cells, during which little activity could be recovered by lysing the cells and assaying for the input DNA. After this time most of the activity had returned and *linkage* had occurred between input and resident markers. Lacks and Hotchkiss[12] found that amylomaltase production started within 10 min after input of the appropriately marked DNA. Later, Lacks[13] found that 50% of the DNA was broken down immediately on entry, while 50% appeared in a denatured form. By 10 min, however, this DNA was back in an apparently native structure, having either induced a specific synthesis of its complement or found a pre-existing complement.

With the present results, we now interpret the phenomenon as follows: on uptake of native DNA, either strand may be broken down, and the other cannot be read for new messages until it is reincorporated into a native-like structure. Expression is therefore the average of that of the two strands. Denatured DNA is less readily taken up by the cell, but that part which is taken up expresses like native DNA. The pH 12 fractionated strands, however, express more or less rapidly, depending on which is present in the fraction, and it appears that they find a pre-existing complement. Otherwise, if they induced synthesis of a new complement, the net result at the end of 10 min would be identical for both strands. Instead, the process appears to be as illustrated in Figure 7, where strand A is the one transcribed directly into messenger RNA. Depending on the extent to which the cells are synchronously dividing, due to their treatment during development of transforming competence,[9] there may be some lag in replication. Incomplete fractionation and a partially synchronous but variable lag in synthesis could account for the plateaus we see often in our curves (e.g., Fig. 5), but more data will be required to give a precise description of these kinetics.

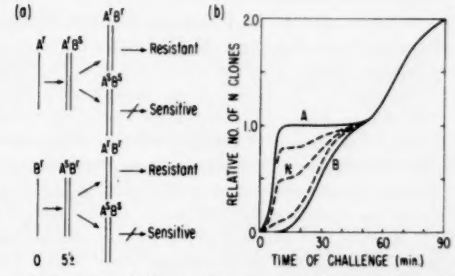

FIG. 7.—(*a*) Apparent course of integration and replication of incoming DNA strands. (*b*) Approximate expression curves for pure fractions, native mixture, and partially fractionated mixtures, according to expectation from (*a*) assuming negligible lag in expression after A^r strand is in 2-stranded helix.

One final point is of interest, namely, that although the RNA polymerase copies only one of the strands into useful message, it does so from a two-stranded structure. *In vitro* experiments show that both strands are copied[14] and useful messenger RNA is produced from a native DNA primer.[15] It is probable that if a signal in the cell (the operator ?) tells the enzyme which to copy, this control has been lost in the test tube. When denatured DNA is used as primer, only a little RNA is synthesized and it is not functional as messenger.[15, 16] We propose that the second strand of DNA has two functions: (*a*) to provide replicas of the A strand, and (*b*) to release the messenger RNA from its template by reforming a preferred

two-stranded DNA. With denatured primer, presumably the messenger sticks to the DNA, and one should be able to find it there.[17]

As for the question of why message RNA compositions are often similar to total DNA composition, even though only one strand is copied, it is clear that since one must look closely to find any heterogeneity of density in denatured DNAs at pH 7 or 12, the composition of most single strands is in fact close to that of the total DNA.[18]

* Supported by a grant (GM-10965-03) from the National Institutes of Health.

† These results were presented at the Federation Meetings, Atlantic City, April, 1963 (Fed. Proc., **22**, Abstr. 2887, 1963).

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[6] In the presence of alkali we have equilibrated up to 0.7 mg DNA without viscosity effects grossly distorting the band profile, whereas with 0.05 mg of native unsheared DNA the profile was in the form of a right triangle, rising from near zero to maximum in 1–2 drops.

[7] For this purpose all $\Delta\rho$'s were calculated from the references as $\Delta\rho = 8.40 \times 10^{-10}\ \omega^2 \bar{r} \Delta r$.[5] By pycnometry, the $\bar{\rho}$'s at pH 12 were all approximately 0.06 g/ml greater than at pH 7, as found by Vinograd *et al.*[1]

[8] Marmur, J., and P. Doty, *J. Mol. Biol.*, **3**, 585 (1961).

[9] Hotchkiss, R. D., these PROCEEDINGS, **40**, 49 (1954).

[10] In some cases, the differences became more marked when the fractions were heated to 100°C after collection. This is to be expected if this treatment reduced the relative activity of spontaneously renatured DNA (therefore containing both strands and with greater activity per unit of DNA) compared to the excess of denatured single strands.

[11] Fox, M. S., *Nature*, **187**, 1004 (1960).

[12] Lacks, S., and R. D. Hotchkiss, *Biochim. Biophys. Acta*, **45**, 155 (1960).

[13] Lacks, S., *J. Mol. Biol.*, **5**, 119 (1962).

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[15] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

[16] Wood, W. B., and P. Berg, these PROCEEDINGS, **48**, 94 (1962).

[17] Since this was written, such a complex has been reported; see Warner, R. C., H. H. Samuels, M. T. Abbott, and J. S. Krakow, these PROCEEDINGS, **49**, 533 (1963).

[18] Marmur and collaborators find that in the DNA of phages alpha and Sp-8 the compositions of the strands differ sufficiently so that they form separate bands in CsCl even at neutral pH. (Marmur, J., and S. Cordes, *Symposium on Informational Macromolecules, Rutgers*, ed. H. Vogel, V. Bryson, and J. O. Lampen (New York: Academic Press, Inc., 1963).

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*GENETIC CONTROL OF A SERUM ESTERASE COMPONENT IN MUS MUSCULUS**

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Communicated by L. C. Dunn, May 27, 1963

The recent coupling of starch-gel electrophoresis[1] and certain histochemical techniques[2] has demonstrated that a considerable number of enzymes exist in a number of electrophoretically separable forms within a single organism.[3] The esterases, which have received considerable attention in the house mouse, *Mus*

musculus, form one of these enzyme complexes. Until recently, these studies have been concerned with the detection and characterization (determining substrates and inhibitors) of the various members,[4,5] finding their distribution in various organs,[6] determining their location in cells,[7] comparing them with similar components of other organisms,[8, 9] and relating changes in the components to various pathological conditions.[10] Except for Popp's recent studies,[11] little is known about the genetic control of the esterases and also other enzyme complexes in this species. As a matter of fact, genetic studies of enzyme complexes in general appear to be few in number, consisting of investigations by Augustinsson and Olsson on piglet arylesterases,[12] by Roderick on polygenic control of rat cerebral cholinesterases,[13] by Allen on *Tetrahymena* esterases,[14] by Bach *et al.* on alkaline phosphatases in *Escherichia coli*,[15] by Schwartz on esterases of maize,[16] and by Tashian and Shaw on erythrocytic acetylesterases in man.[17]

An additional genetic control of the serum esterases in *Mus musculus* has recently been described.[18] In the present paper, data are presented which suggest a one-locus, two-allele, mode of inheritance of the fastest migrating, anodal band in the serum esterases.

Materials and Methods.—Representatives of a number of laboratory strains, including C3H, DBA/2, C57B1/6, BALB/C, SEC/Re, CBA, YBr, and AT, as well as three feral populations of the house mouse (Washtenaw County, Michigan) were used. Because of changes in the serum esterase patterns during maturation, the mice were not bled until they were at least seven weeks old, an age at which the patterns appear stable.

Blood was obtained from the mice by inserting a 1.5–2.0 mm capillary tube into the suborbital canthal sinus. The sample was centrifuged at room temperature immediately after being withdrawn, and was stored overnight at 4°C. Approximately 10 lambda of serum were placed on a 5 × 10 mm strip of Whatman No. 1 filter paper, which was then inserted into a slit in a 120 × 48 mm starch-gel slab. A minimum of three samples were run side by side. During the horizontal electrophoresis a voltage drop of 6 v/cm was maintained along the starch-gel for a period of 5 hr at room temperature. The bridge and gel buffers were, respectively, 0.3 and 0.03 molar borate, with a pH in each case of about 8.5. After electrophoresis, the starch-gels were sliced into upper and lower halves, and incubated for 30 min at 37°C in an α-naphthyl butyrate diazonium mixture composed of: 100 mg of fast blue RR salt (stable diazotate of 4′-amino-2′,5′-dimethoxybenzanilide), 180 ml of distilled water, 16 ml of 0.1*M*, pH 6.8 phosphate buffer, and 4 ml of 1% acetone solution of α-naphthyl butyrate.

Several runs were also made with α-naphthyl acetate as the substrate.

Results.—The serum esterase zymograms of the laboratory mice appeared highly uniform with the procedure utilized, except for the variant in the C57B1 strain described by Popp[11] and slight variations in staining intensities. These zymograms revealed two bands in addition to the nine already described by Hunter and Strachan.[4] For the purpose of this study these 11 bands have been numbered I to XI, beginning with the fastest anodally migrating member (see Fig. 1*a*).

In the initial survey of seven feral mice, six showed zymograms similar to those of the laboratory strains. The zymogram in the remaining animal, a pregnant female, revealed that the fastest migrating serum esterase band, or *serum esterase I*,

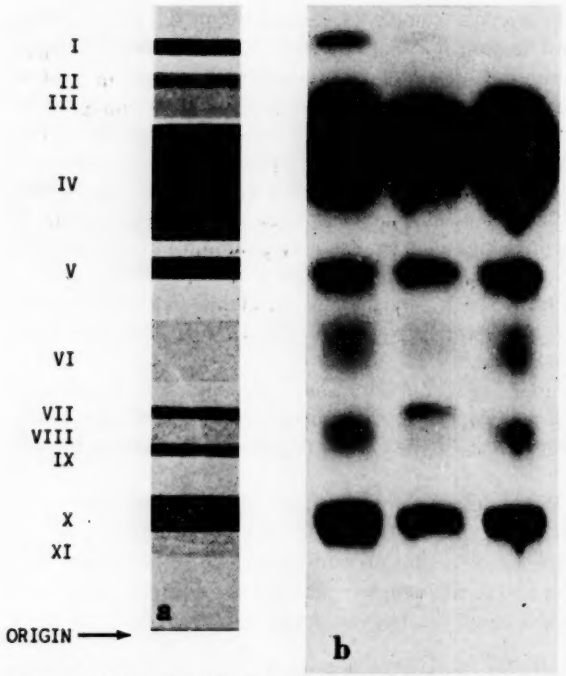

FIG. 1.—(*a*) A composite schematic representation of the serum esterase starch-gel electrophoretic patterns of the house mouse, *Mus musculus*, after incubation with alpha-naphthyl butyrate; (*b*) a zymogram showing the three phenotypes of the fastest migrating serum component (*band I*). The respective genotypes are, from left to right, *Es-2*^{b}/*Es-2*^{b}, *Es-2*^{a}/*Es-2*^{b}, and *Es-2*^{a}/*Es-2*^{a}.

was missing. One of the four progeny, all females, of this pregnant female also lacked the first band. The remaining three had a light first band. The offspring lacking this first band was then mated to a C3H male. C3H mice have a prominent *esterase I*. All of the F_1's had a *serum esterase I* which was considerably lighter than that of the C3H sire. The F_2's were classified into three types on the basis of the *serum esterase I:* those lacking the *serum esterase I*, those having a light first band, and those in which this fastest migrating band was prominent (see Fig. 1*b*). Subsequently, the F_2's were used in making the matings which are summarized in Table 1.

Zymograms, in which the substrate was α-naphthyl acetate, although similar to those prepared with α-naphthyl butyrate, were not as clear.

Aside from the variation in *serum esterase I*, study of a larger sample of individual feral mice has revealed differences in some of the other bands. For instance, *bands II, III, VII,* and *IX* are sometimes found to be missing. *Serum esterase V*, which usually appears as a single band, has on occasion been found to be doubled. Also, differences in migration rate of *serum esterase X* have been noted in a few animals.

TABLE 1

SUMMARY OF MATING-OFFSPRING *Serum Esterase I* PHENOTYPES IN *Mus musculus*

Mating type code	*Esterase I* phenotype of mating	No. of sib-ships	No. of individuals	Offspring Phenotypes: dark	light	absent	X^2	*d.f.*	*P**
1	dark X dark	8	42	42	0	0	...		
2	absent X absent	6	38	0	0	38	...		
3	absent X dark	7	43	0	43	0	...		
4	light X light	13	109	28	60	21	2.009	2	0.30–0.50
5	light X dark	14	108	57	51	0	0.2315	1	0.50–0.70
6	light X absent	11	66	0	35	31	0.1364	1	0.70–0.80
	Total	59	397						

Chi-square tests are based on the hypothesis of a single-locus, two-allele, mode of inheritance.

Discussion.—The results of all matings involving the three *serum esterase I* phenotypes are consistent with the hypothesis that this esterase component is under the control of a pair of incompletely dominant alleles at a single autosomal locus. For example, when the results of matings between animals both of which had a light first band (mating type 4), between animals where one had a dark *serum esterase I* and the other had a light *serum esterase I* (mating type 5), and between animals in which one had a light *serum esterase I* and the other lacked the first band (mating type 6), were tested against 1:2:1, 1:1, and 1:1 ratios, respectively, chi-square values showed a good fit between observed and expected phenotypes. Also, when both parents had either a dark *serum esterase I* band or lacked this band altogether, the offspring were of the parental phenotype (mating types 1 and 2). Finally, when one parent lacked the *serum esterase I* and the other had a dark first band (mating type 3), all the offspring were of the intermediate phenotype.

The locus controlling these *serum esterase I* types is tentatively designated *Es-2* in view of (1) the lack of any knowledge of the specific function of this component, (2) Popp's *Es* designation of another locus affecting mouse serum esterases,[11] and (3) the need of a system permitting maximum flexibility in designating esterase loci to be discovered in the future. The alleles are designated by letter superscripts, as recommended by rules set forth by the Committee on Standardized Genetic Nomenclature for Mice.[19] $Es\text{-}2^a$ is therefore the symbol for the allele resulting in the nonexpression of *serum esterase I*, and $Es\text{-}2^b$ represents the other allele at this locus. Thus, the three phenotypes evident in the zymograms (no apparent first band, light first band, and dark first band) correspond, respectively, to the genotypes $Es\text{-}2^a/Es\text{-}2^a$, $Es\text{-}2^a/Es\text{-}2^b$, and $Es\text{-}2^b/Es\text{-}2^b$.

It is not yet known to what extent modifications in serum esterase components, other than the first and the fourth (Popp's), are under genetic control. Preliminary breeding data suggest that the variations seen in *band VII* may be due to alleles at a locus other than *Es* and *Es-2*.

A preliminary study has revealed that the *esterase I* band is absent not only in the serum of $Es\text{-}2^a/Es\text{-}2^a$ animals but also in homogenates of liver, kidney, and duodenum, three tissues which usually demonstrate considerable *esterase I* activity. These findings suggest that the *Es-2* locus affects the function of the *esterase I* component, not only in the serum but also in all tissues of the body. A similar situation has been found in the liver and kidney zymograms of several mice by Paul and Fottrell.[9]

The results of the present study are consistent with the conclusions reached by Hunter and Strachan, who, after using a number of substrates and inhibitors, decided that "it is unlikely that the esterases of mouse blood can be subdivided into groups sharing properties which would warrant their classification into isozymes."[4] The change in the appearance of *band I* was not associated with any other modification of the esterase zymogram. If the *esterase I* component were a member of an isozymic group, then the locus which controls this component would also be expected to affect the appearance of other esterase components. Since there is no evidence of this occurring, the locus is presumably concerned only with this one esterase component. Moreover, the present data are also in agreement with Popp's contention that the molecular structure of each component of the serum esterase complex is determined by a separate locus.[11]

Summary.—Sera from house mice of eight laboratory strains and three feral populations have been examined for esterase activity by means of starch-gel electrophoresis. In one of the feral animals (a pregnant female) a modification in the first anodal band (*esterase I*) was observed. Results obtained from mating the progeny of this female were consistent with the hypothesis that the appearance of this first esterase band is under the control of a pair of autosomal alleles, lacking dominance. The locus has been designated *Es-2* with alleles *Es-2*^{a} and *Es-2*^{b}. Evidence was obtained which suggests that the *esterase I* component is not a member of an isozymic group.

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*DYNAMICS OF CHOLESTEROL METABOLISM, I. FACTORS REGULATING TOTAL STEROL BIOSYNTHESIS AND ACCUMULATION IN THE RAT**

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During the course of steroid balance studies in rats, it was observed that total animal sterol synthesis was nearly proportional to caloric intake and appeared independent of several experimental dietary changes. Total animal sterol synthesis was also noted to be independent of dietary sterol levels at the lower levels found in ordinary laboratory diets, implying that cholesterol feed-back inhibition of its own synthesis[1] may not be significant under such dietary conditions. These findings form the basis of this report.

Methods.—Animals and diets: Data was collected from 38 male Wistar rats over a 15 week period. This included a 2 or 3 week control period, during which animals were fed laboratory chow, and a 12 week experimental dietary period. The animals were divided into six groups, each group named according to its principal diet. Diets A, E, and F had a dietary base of natural products blended to a laboratory chow. Diets B, C, and D had a synthetic dietary base of casein, sucrose, and cellulose. Caloric equivalents were calculated from manufacturer's specifications. The experimental diet contents are detailed in Table 1. Caloric intake among the groups was variable since differences in appetite, diet acceptance, and diet absorption were observed. Diet C was modified 4 times before it was satisfactorily accepted. These differences in caloric intake were reflected in growth (Fig. 1). Animals were chloroformed during the 15th week, and tissues were processed immediately or frozen. Carcasses were weighed, minced, diluted with water to 1,000 gm, and blended mechanically. Weighed aliquots were taken for analysis.

Fecal sampling: Feces were collected on aluminum screening and analyzed weekly. Fecal pellets were air-dried for 3 days after being separated manually from spilled food and hair. This type of collection was used because total fecal steroids, independent of minor oxidative changes,[2] were being analyzed. The dried pellets were ground to a fine uniform powder in a mechanical blendor and two gm were taken for analysis.

TABLE 1

SUMMARY OF EXPERIMENTAL DIETS

Name of diet	Basic Ingredient		Added Drug or Food		Dietary Composition (per cent by weight)				Dietary Sterol mg/gm	
	Source	%	Name	%	Protein	Carbohydrate	Fat	Calories per 100 gm	Total sterol	Cholesterol
A	RM*	100	None	—	24.3	50.0	4.15	335	1.06	0.23
B	NBC-1†	100	None	—	20.4	57.0	2.44	332	0.06	0.06
C-1	NBC-2‡	100	None	—	20.0	38.0	30.0	502	0.04	0.04
C-2	Diet C-1	67	NBC-3§	33	21.7	47.0	20.0	455	0.05	0.05
C-3	Diet C-2	50	Diet B	50	21.0	52.0	11.2	393	0.06	0.06
C-4	Diet C-3	50	Diet B	50	20.7	54.5	6.8	362	0.06	0.06
D	Diet B	98	Nicotinic acid	2	20.0	56.0	2.40	326**	0.06	0.06
E	Diet A	80	Lard	20	19.2	40.0	23.2	446	0.98	0.22
F	Diet A	80	Stearic acid	20	19.2	40.0	23.2	446	0.96	0.21

* Rockland mouse diet, A. E. Staley Mfg. Co., Decatur, Illinois. Fatty acid content measured 36.8 mg/gm and showed by gas chromatographic analysis; 14:0—1.6%; 16:0—20.0%; 16:1—2.4%; 18:0—7.3%; 18:1—31.8%; 18:2—34.4%; 18:3—2.3%.

† Nutritional Biochemical Co. (NBC), Cleveland, Ohio. "Fat-free" test diet with essential fat added as "methyl linoleate—75%." Gas chromatographic analysis of this fat source showed the following fatty acid content: 16:0—7.1%; 18:0—2.9%; 18:1—13.0%; and 18:2—77.0%.

‡ NBC "fat-free" test diet modified to contain 20% protein and 30% "methyl linoleate—75," gift of Nutritional Biochemical Co.

§ NBC "cholesterol-free" test diet.

** Assumes no caloric value for nicotinic acid.

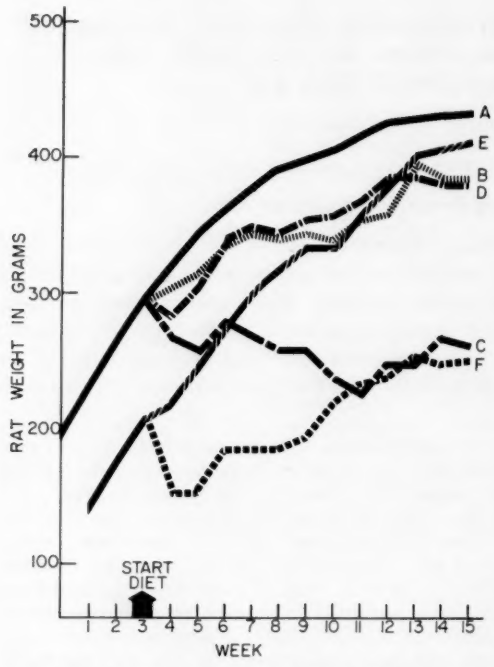

FIG. 1.—Average growth curves for each of the 6 groups of rats. Experimental diets were begun in the fourth week (arrow).

Extractions and analyses: Carcass aliquots and samples of diets were extracted with acetone-ethanol (1:1).[3] After hydrolysis and digitonin precipitation[4] they were analyzed for total fast- and slow-acting sterols.[5] Dietary sterols were also analyzed by gas-liquid chromatography.[6] Feces were extracted from 50 vol acetone:alcohol, 1:1, V/V, refluxed at ebullient temperature for 3 hr. Aliquots of the lipid extracts were reduced under nitrogen to 2–5 ml and saponified for 1 hr over steam in 10 ml of 80% ethanol and 1 *N* potassium hydroxide. Then 20 ml distilled water was added to each sample, and nonsaponifiable lipids were extracted with hexane. Six hexane extractions were necessary for uniform and reproducible results.[7] The nonsaponifiables were washed with distilled water, dried over sodium sulfate, and filtered. The filtrate contained "total fecal sterols." The aqueous residual was acidified with 8 *N* sulfuric acid to pH 1. Acidic lipids were extracted with three ether extractions. The ether extract was washed with saturated sodium chloride until neutral, dried over sodium sulfate, and filtered. The extract contained "total fecal bile acids."

Analysis of fecal sterols and bile acids: "Total fecal sterols" were analyzed as fast- and slow-acting sterols by the Liebermann-Burchard color method of Moore and Baumann.[5] During the first and second weeks and from the tenth to twelfth week selected samples of feces were analyzed by 4 different methods on hexane and/or ether extracted nonsaponifiables (Table 2). The four methods were: (1) the Liebermann-Burchard color method (LB);[5] (2) gas-liquid chromatography (GLC);[6,8] (3) gravimetric analysis of sterol digitonides; and (4) LB color method on precipitated digitonides. The consistent agreement of the LB color reaction from hexane extracted material with isotopic data (see below) and with data obtained by the more laborious methods of gas chromatography and gravimetric analysis of precipitated digitonides made it the method of choice for sterol determinations. "Total fecal bile acids" denotes results obtained by application of the fluorimetric method of Levin *et al.*[9] to the acidic lipid extract described above.

Isotope studies: Isotopic data following injection of purified[10] cholesterol 4-C^{14} corroborated chemical data. During the tenth week, three animals from groups A, B, D, E, and F were in-

TABLE 2

COMPARATIVE RESULTS OF TOTAL FECAL STEROLS IN RAT FECES DETERMINED BY 4 METHODS

Diet	Week	No. of samples	Non-saponifiables extracted with:	Total Sterols as Mg per 2 Gm Rat Feces by: L-B color	Gas chromatography	Digitonin ppt. (gravimetric)	Digitonin ppt. (L-B color)
A	2	3	Hexane	6.7	6.9	6.7	3.1
A	2	3	Ether	8.7	6.5	7.2	3.8
B	10–12	7	Hexane	4.3	3.6	4.8	2.6
C	10–12	9	Hexane	5.9	6.8	7.7	4.0
D	10–12	9	Hexane	5.6	5.4	6.0	3.4
E	10–12	6	Hexane	7.9	7.7	9.8	5.7
F	10–12	6	Hexane	4.7	4.4	6.0	2.8

jected intraperitoneally with 1.76×10^6 cpm of tracer cholesterol 4-C^{14} in 0.5 ml 1% bovine serum albumin in 0.85% saline solution. Radioactivity in fecal bile acid and sterol fractions was analyzed weekly. Isotopic fecal bile acid:sterol ratios were compared with chemical ratios as a check on the chemical methods.

Calculation of sterol synthesis by the balance method: Total body sterol synthesis was calculated from sterol intake and output (as fecal sterols and bile acids) and changes in total body sterol content. It was assumed that significant sterol degradation does not occur. Total body sterols were calculated at the beginning of the dietary study by analysis of 4 control rats and at the end by analysis of the subject animals. Changes in body sterol were assumed to be a straight line function with respect to time (Fig. 2). While this assumption may not be strictly correct, it can be seen from Table 3 that the weekly changes assumed for body sterol content were small compared to total animal sterol synthesis. The equation for calculating the sterol synthesis data is $S_T = \Delta s + Fs + Fba - Ds$, where S_T is the total sterol synthesized (column 9), Δs is the change in total body sterol (column 6), Fs is total fecal sterol (column 7), Fba is total fecal bile acids (column 8), and Ds is dietary sterol (column 5).

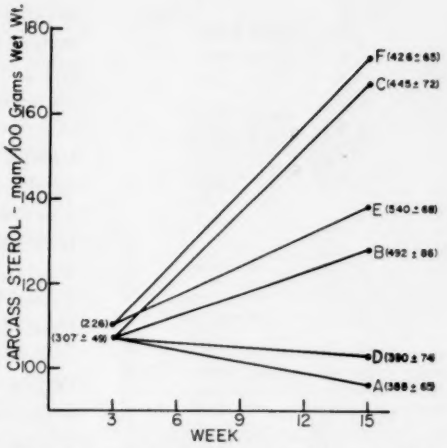

FIG. 2.—Changes in carcass sterol concentration from weeks 4–15. Figures in parentheses are averages of total carcass sterol for each group at time of sacrifice.

Results.—The most significant results of this study are summarized in Table 3 which represents a 7 week steady state period. The term "steady state" means a steady dietary state during which dietary intake and sterol biosynthesis were relatively level within each group.

Sterol synthesis in the normal 400 gm rat fed laboratory chow during the steady state period varied 183–238 mg per rat per week. Total sterol synthesis was roughly proportional to caloric intake within all groups. Figure 3 is a plot of the 72 individual mean weekly values for each group during the 12 week experimental diet period. Figure 4, a plot of the average values measured during the steady state period, is based upon 40 average values which show a slope of 0.40 mg sterol synthesis per calorie and a correlation coefficient of 0.77.

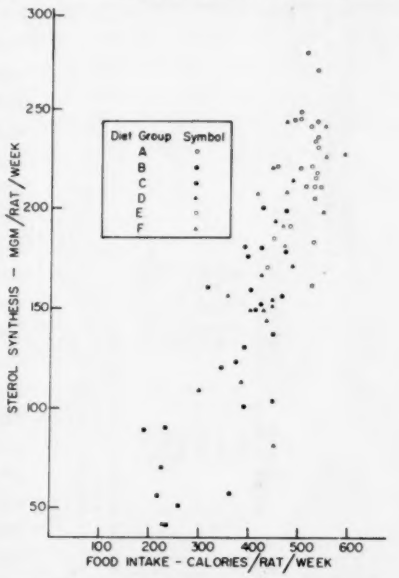

FIG. 3.—Spot diagram of weekly average values of sterol synthesis and caloric intake during entire experimental dietary period (weeks 4–15).

Sterol synthesis and dietary sterol: It might be assumed from published data con-

TABLE 3

SUMMARY OF STEADY STATE BALANCE DATA
(ACCUMULATED FROM 6TH THROUGH 12TH WEEK)*

1	2	3	4	5	6	7	8	9	10
Diet	No. animals	Average weight in grams†	Calorie intake calories/rat/week	Total sterol intake mg./rat/week§	Increase in body sterol mg./rat/week	Total fecal sterol mgm./rat/week	Total fecal bile acid mgm./rat/week	—Total sterol synthesis— mgm./rat/week	mgm./calorie
A	4	396	524 (13.2)‡	168	9	158 (16.4)	221 (16.5)	220 (28.4)	0.42
B	4	345	402 (28.6)‡	7	13	45 (6.4)	103 (13.8)	156 (26.5)	0.39
C	7	253	261 (62.6)	4	11	41 (9.2)	39 (9.6)	87 (40.8)	0.33
D	8	356	440 (43.7)	8	9	56 (6.7)	114 (11.0)	171 (22.8)	0.39
E	5	325	526 (10.2)	115	28	121 (12.3)	187 (19.4)	221 (33.2)	0.42
F	6	227	542 (37.3)**	135	32	130 (5.1)	200 (17.8)	228 (26.1)	0.38

* Diet F data tabulated for 8th through 12th week.
† Mean animal weights at start of diets, i.e., end of week 3; diets A–D 297 gm: diets E & F, 198 gm.
‡ Figures in parentheses represent standard deviations of the means.
§ Gas chromatographic analysis showed the dietary sterol of diets A, E, and F included 22% cholesterol, and diets B, C, and D 100% cholesterol.
** This figure derived by assuming *absorption* of stearic acid is 66% as indicated by fecal stearic acid titration data.

cerning cholesterol feed-back inhibition of its own synthesis[1, 11–14] that an animal normally fed a diet containing some cholesterol would increase its sterol synthetic capacity if that diet were changed to one containing less cholesterol. At the low levels of dietary cholesterol concentration in these experiments,[14a] this was not the case. For example, Group B animals had consistently lower rates of sterol synthesis than did Group A. The differences were determined by dietary factors other than cholesterol.

Accumulation of sterol in carcass: Concentration of sterol in carcass is seen in Figure 2 which assumes carcass sterol concentration is representative of the whole animal, and changes in carcass sterol are linear with respect to time. Changes in body sterol in mg per rat per week (Column 6, Table 3) were interpolations from Figure 2. General features noted from these data are: (1) Animals which lost weight and failed to grow (diets C and F) had higher concentrations of sterols in carcass than controls. (2) The variations in acceptance, absorption, and excretion between animals on different diets must be considered when comparisons are made. (3) Dietary bulk, or fecal residue, appears to be inversely related to sterol accumulation. Carcasses from low bulk, low residue, synthetic diet B animals had generally higher sterol concentrations and total body sterol accumulations than did control animals on laboratory chow (diet A). When chow-based diets A and E are compared, there are similar and striking differences. Both groups ate and grew at comparable rates throughout the study. The average weekly dry weight of feces for groups A and E was 46.5 and 30.0 gm, respectively. Diet E, the well-tolerated diet rich in saturated fat and yielding less fecal bulk, led to both increased concentration of carcass sterol and increased total carcass sterol, although it contained less sterol than diet A, and the animals which ate it synthesized sterol at the same rate as did those with diet A. It can be seen from Table 3, columns 7 and 8, that the diet A animals consistently excreted 10 to

20 per cent more fecal sterols and bile acids than did the animals fed diet E. Other groups are more complicated to compare because of disparities of growth and caloric intake. The Group C animals accumulated almost as much total sterol (Fig. 2) as did their better fed controls (Group B), despite poor diet acceptance and a highly unsaturated diet. A major factor involved appeared to be the small amount of fecal residue and steroids.

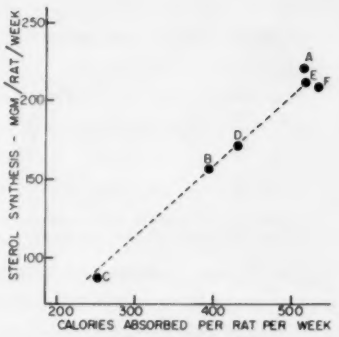

FIG. 4.—Relationship of caloric intake to total animal sterol synthesis during steady state period (weeks 6–12). Values for each group are averages taken from columns 4 and 9 of Table 4.

Figure 5 is a plot of feces weight versus total fecal bile acids during the steady state period. Bile acid excretion was closely related to the weight of fecal residue. The same was true of fecal sterol excretion when the synthetic and laboratory chow diets were compared (Table 3). These data are consistent with the observation that carcass sterol accumulation is inversely related to fecal bulk. A determining factor in carcass sterol accumulation lies in the ability of the animal to excrete its own sterol either as sterol or as bile acid. It would seem from these data that dietary bulk is a major medium for transporting steroids from the animal body.

Isotope studies: Isotopic data following injection of cholesterol 4-C^{14} corroborated conclusions from the chemical balances. The radioactivity of excreted fecal products paralleled closely and confirmed the chemical results. Table 4 shows results for a period when all animals were in a steady state, and illustrates the similarity between chemical and isotopic data. The fecal bile acid to sterol ratio was higher for synthetic diets B and D than for laboratory chow diets A, E, and F. The consistency of these ratios was borne out by analysis of the chemical figures from weeks 4–14.

Discussion.—This work confirms several lines of investigation by others. Previous workers have shown *in vitro*[15] and *in vivo*[16] that fasting diminished cholesterol biosynthesis and that the cholesterol biosynthetic pathway is energy-dependent.[3a, 17] It is not surprising that sterol synthesis is calorie-dependent. The mature animal in a steady state of sterol balance accounts for his entire sterol synthesis by G. I. tract turnover (excluding urinary and skin losses). It seems possible that fecal steroid excretion somehow affects sterol synthesis. The rate of removal of steroids from the body by fecal residues correlates with the rate of body sterol synthesis. It is as if an inhibitor of sterol synthesis were being removed in the feces, and parallels the work of Thompson and Vars,[18,19] and Eriksson[20] who showed that bile

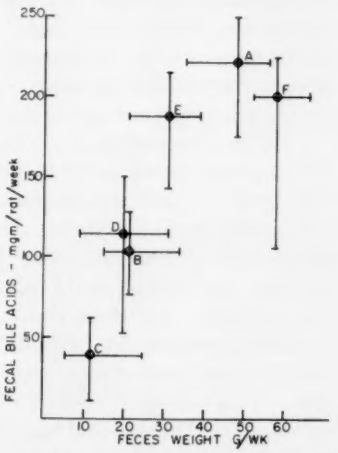

FIG. 5.—Relationship between total fecal bile acids and air-dried feces weight during steady state period. Vertical and horizontal lines give range of values for each point.

TABLE 4

COMPARISON OF BILE ACID TO STEROL RATIOS DERIVED FROM CHEMICAL AND ISOTOPIC DATA FOR RATS ON VARIOUS DIETS*

Diet	Bile acid:sterol ratios (Weeks 11 and 12)		
	Chemical	C^{14}	Average
A	1.44	1.32	1.38
B	2.41	2.14	2.27
D	1.90	1.82	1.86
E	1.58	1.54	1.56
F	1.55	1.56	1.56

* Figures are averages of simultaneous chemical and radioactivity determinations of six animal weeks for each diet during weeks 11 and 12 of the study.

acid synthesis increased 10-fold in bile fistula rats. The animal with a high residue diet acts similarly to the bile fistula animal, and it might be said that it has an internal form of bile fistula. Portman and Murphy[21] had similar results when they emphasized the effect of diet on the excretion of fecal metabolites of cholesterol.

Bile acids are known to influence cholesterol synthesis[22,23] and it might be speculated that it is the bile acids being removed in the feces that are the normal physiological inhibitors of cholesterol biosynthesis. It is probable that bile acids which inhibit sterol biosynthesis must be unconjugated, since bile duct ligation, followed by elevations in serum *conjugated* bile acids, leads to increased rates of cholesterol biosynthesis.[24] One may question whether cholesterol itself is truly the inhibitor of its own biosynthesis as demonstrated by feeding experiments. Another explanation might be that some cholesterol in transit through the gut is modified to a steroid acidic product which inhibits cholesterol biosynthesis.

From these ideas an over-all concept of body sterol metabolism in terms of biosynthesis, accumulation, and excretion can be envisaged. Total animal sterol biosynthesis is regulated by three physiological factors: (1) *caloric intake* which provides the necessary energy for sterol synthesis; (2) *fecal steroid excretion* which is postulated to regulate sterol biosynthesis by influencing the reabsorption of unconjugated bile acids. These acids are postulated to be prime physiological inhibitors of sterol biosynthesis. Fecal steroid excretion is in turn proportional to fecal residue or bulk; (3) *dietary cholesterol* which when fed in higher concentrations (approaching the 0.5 per cent level) also regulates sterol biosynthesis, although the mechanism may be similar to that controlled by fecal steroid excretion.

Total sterol accumulation in an animal fed a calorically adequate, low cholesterol diet is primarily regulated by fecal steroid excretion, and this is proportional to fecal residue. Although increased fecal steroid excretion promotes higher rates of endogenous sterol biosynthesis, the dominant effect observed is lesser body sterol accumulation. Similarly, when fecal steroid excretion is reduced by a low residue diet and total animal sterol biosynthesis falls, body sterol accumulation nevertheless increases. For these reasons, animal sterol accumulation appears to be less dependent upon the rate of total animal sterol biosynthesis than upon the differential rates between absorption of exogenous sterol and excretion of steroid in dietary residue. This concept is entirely consistent with the work of Steiner *et al.*[25] and Manalo *et al.*[26] in humans who have challenged the concept that the diminished hepatic cholesterol biosynthesis found after cholesterol feeding can adequately compensate for increased absorption of excess dietary cholesterol.

The rates of total sterol synthesis presented are similar to those found by others when the specific dietary conditions of individual studies are noted. Pihl *et al.*[27]

estimated that the half time of carcass sterol turnover was 31–32 days in rats fed 321 calories per week. Gould and Cook[3b] estimated that the rate of synthesis in a 300 gm rat would be 14 mg per day or 98 mg per week. These values fall within the spread observed in Figure 3. Lindstedt and Norman,[28] feeding rats white bread and water, arrived at a slightly lower figure for total sterol synthesis of 9–12 mg per 300 gm rat, but the caloric intake was not recorded.

Nicotinic acid (Group D) was not found to influence significantly total sterol synthesis accumulation, or excretion in these studies.

Summary.—Balance studies in adult male rats have shown that total animal sterol synthesis was proportional to caloric intake. Total body sterol biosynthesis was independent of variations in cholesterol feeding levels when these levels remained in the range of 0.006–0.023 per cent by weight. Fecal dietary residue, as measured by air-dried weight, was also related to total animal sterol synthesis and inversely related to the accumulation of sterol in the animal carcass. A cholesterol-accumulating, or atherogenic, diet is one which is high in calories, well refined, and low in fecal residue. Such a diet would contain a high fat content to provide plentiful calories with minimal residue and optimum conditions for cholesterol absorption and reabsorption.

This work was completed during the tenure of an Established Investigatorship of the American Heart Association. It was supported by grants from Heart Societies of Cleveland, Portage County, and Western Counties of Ohio, and by U.S. Public Health Service grant A-5304. The technical assistance of A. P. S. Dhariwal and Miss Carole Goldman is gratefully acknowledged. Quantitative analysis of dietary fatty acids by gas chromatography was kindly performed by William Insull, Jr.

* Definition of terms to be used in this paper: *Sterol*—a neutral lipid of the cyclopentanophenanthrene series; *Bile acid*—an acidic lipid of the same series; *Steroid*—inclusive term for sterols and bile acids; *Sterol synthesis*—the creation of a sterol or bile acid from a nonsteroid precursor, presumably acetate. Bile acid synthesis is included in the term sterol synthesis, since cholesterol is generally accepted as an intermediate.

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[14a] Diet A contained 1.06 mg total sterol per gm of diet. Of this, 22%, 0.23 mg per gm diet, was cholesterol as determined by gas chromatographic analysis. This dietary cholesterol level of 0.023% by weight must be compared with experiments showing cholesterol feed-back inhibition where cholesterol has been fed at levels of 0.5–5%[1,11–14] or 20–200 times the level present in

ordinary laboratory chow. Diet B, supposedly cholesterol-free, contained 0.06 mg cholesterol per gm of diet, or about one fourth the cholesterol content of diet A.

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*ON THE CONSTRUCTION OF CERTAIN LIAPUNOV FUNCTIONS**

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Communicated by Marston Morse, June 3, 1963

This note is a brief account of a longer paper which will be published elsewhere.[1]

Let $r(x,y)$ be a function of class C' neighboring (0,0) with $r(0,0) = 0$, and consider the differential equation

$$\ddot{x} = r(x,\dot{x}). \tag{1}$$

With (1) we associate the system

$$\dot{x} = y, \tag{2}$$

$$\dot{y} = r(x,y).$$

The system (2) is said to be *regular* near (0,0) if $r_y(0,0) \neq 0$.

LEMMA. *If the system* (2) *is regular and has the origin as an isolated singular point, the function* $V(x,y)$ *defined by the equation*

$$2V(x,y) = y^2 - 2\int_0^x r(x,0)dx$$

is a "Liapunov" function which determines the stability or instability of the origin.

Specifically, this function V may be employed in conjunction with well-known theorems of Liapunov, Cetaev, and LaSalle-Lefschetz to determine the nature of the critical point at (0,0) [see ref. 2].

THEOREM 1. *If the origin is an isolated critical point of a regular system* (2), *this point is (locally) asymptotically stable if*

$$r_y(0,0) < 0 \text{ and } xr(x,0) < 0 \ (x \neq 0).$$

In all other regular cases it is unstable.

The analysis which leads to Theorem 1 also provides the following result.

THEOREM 2. *If the origin is an asymptotically stable isolated critical point of a system* (2), *let N be the origin plus any set of points* (x,y) *throughout which both* $r_y(x,y) < 0$ *and* $xr(x,0) < 0$. *Further, let*

$$k_1 = \underset{(x\in N, x<0)}{l.u.b.} \left[-2\int_0^x r(x,0)dx\right], \quad k_2 = \underset{(x\in N, x>0)}{l.u.b.} \left[-2\int_0^x r(x,0)dx\right],$$

$$k_0 = \min(k_1,k_2).$$

Then the region

$$y^2 - 2\int_0^x r(x,0)dx < k_0$$

is a region of asymptotic stability of the origin.

Among other results in the paper is the construction of a Liapunov function for a differential equation

$$\dddot{x} + \psi(x,\dot{x})\ddot{x} + \theta(x,\dot{x}) = 0, \tag{3}$$

which appears to be the most general third-order (autonomous) equation for which such a construction has been made. We assume that $\theta(0,0) = 0$, $\psi(x,y)$, $\theta(x,y)$ are of class C', and that $\psi_{xx}(x,y)$ and $\theta_{xx}(x,y)$ are continuous near (0,0). Using the Liapunov function

$$2V = z^2 + 2\int_0^y \theta(x,y)dy + 2\alpha\left[yz + \int_0^z \theta(x,0)dx + \int_0^y y\psi(x,y)dy\right],$$

where α is a constant, we prove the following result.

THEOREM 3. *Suppose* $\theta_x(0,0) \geq 0$, $\theta_y(0,0) > 0$, $\alpha = \theta_x(0,0)/\theta_y(0,0)$, *and* $\psi(0,0) > \alpha$. *If* $\psi(x,y) \geq \psi(0,0)$, *and if either* $\dot{V} \equiv 0$ *or* $\dot{V} \not\equiv 0$ *along every nontrivial solution of the associated system*

$$\begin{aligned} \dot{x} &= y, \\ \dot{y} &= z, \\ \dot{z} &= -z\psi(x,y) - \theta(x,y), \end{aligned} \tag{3}'$$

and, finally, if near the origin

$$\theta_x(x,y) + \alpha[y\psi(x,y) - \theta_y(x,y)] \leq 0,$$

the origin is an asymptotically stable critical point of the system (3)'.

The analysis establishing the above results, the construction of Liapunov funotions for some other types of systems, and more complete references will be found in the paper of which this is a brief account.

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*INTRACHROMOSOMAL EXCHANGE AT THE BAR LOCUS IN DROSOPHILA**

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The A^b complexes in maize[1–3] are adjacent, serial duplications whose members have retained synaptic homology and carry the phenotypically distinctive alpha and beta alleles. Certain of the alpha isolations from these complexes are anomalous in that they occur as nonrecombinants for marker loci, and thus are not ascribable to conventional crossing over. Our analyses[4, 5] indicate that these alpha isolates are not the result of gene mutation of the adjacent beta element, or of multiple exchanges in short segments. Rather, they indicate that the nonrecombinant alpha occurrence involves a highly specified, physical loss of the adjacent beta member and that the homologue need not participate in the event.

The mechanism proposed[5, 6] to account for these losses of beta assumes that when pairing forces are initiated at meiosis, the adjacent members of the duplication may either (*a*) engage their counterparts in the homologue in regular or oblique synapsis, or (*b*) pair with each other intrachromosomally to form a double-loop configuration. In the latter case, an exchange between paired members would result in the loss or gain of one complete member of the duplication, depending on the strands involved. The consequences of this event in the case of A^b would be the occasional appearance of alpha strands that are nonrecombinant for markers among other alpha derivatives that result from crossing over in legitimately synapsed homologues and thus appear as recombinants.

The bar duplication in *Drosophila melanogaster* affords a critical test of intrachromosomal recombination. As identified cytologically,[7, 8] bar represents a tandem, serial repeat of the seven bands of the 16A subdivision of the X-chromosome, and Sturtevant's[9] analysis of unequal crossing over at the locus, in the light of this finding, can only mean that the 16A members of the duplication engage in oblique synapsis with counterparts in the homologue, and hence that the adjacent members are themselves synaptically equivalent.

As shown in Figure 1, which illustrates intrachromosomal association of the 16A members of bar, exchanges posed at the chromatid level and occurring within the double loop should lead to the removal or addition of one complete 16A member, no more, no less. At the phenotypic level these exceptions are expected to appear as strands that are nonrecombinant for outside markers. Moreover, analyses of salivary gland chromosomes of progeny of exceptional individuals should afford a precise determination of the extent of these changes at the cytological level.

Materials and Methoas.—In the experiments reported here, bar-locus changes from B to B^+ (bar to normal), B to BB (bar to double-bar), BB to B (double-bar to bar), and BB to B^+ (double-bar to normal), were sought among male, or where feasible, among male and female offspring of appropriately marked mothers. A map of a portion of the X-chromosome spanning the bar locus is given below:

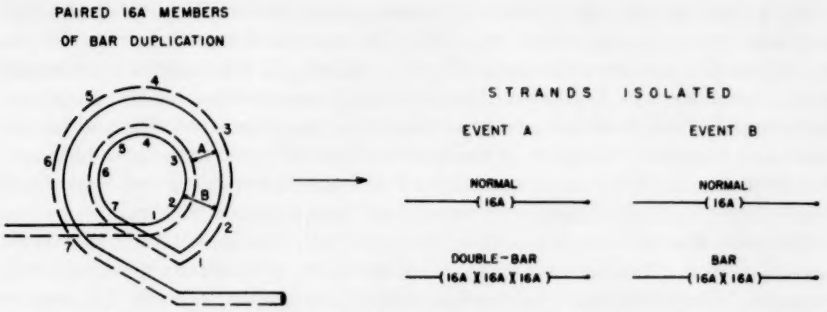

FIG. 1.—Diagrammatic representation of intrachromosomal exchange. At the left, adjacent 16A members of the bar duplication are shown paired with each other at meiosis to form the double loop. Exchanges within this double loop, posed at the chromatid level, lead to single-membered (wild-type) strands and triplication (double-bar) strands, as shown at the right. Since only a single chromosome is involved, exceptional strands are nonrecombinant for marker loci.

Parental females, except as noted otherwise, were heterozygous for *f* (forked bristle) and either *fu* (fused vein) or *od* and *sy* (outstretched wing; small eye), the latter being designated hereafter as *odsy*. Females were mated singly with $f^+ B^+$ *odsy*$^+$ *fu*$^+$ males carrying autosomal markers as a check on contamination, as well as a sex-linked recessive marker to permit identification of patroclinous male offspring. Presumptive exceptional offspring were given appropriate progeny tests to (*a*) confirm legitimacy of the male parent, (*b*) verify exceptional status of the case, and (*c*) identify the exceptional strand as recombinant or nonrecombinant for the marker loci. Nonrecombinant exceptions were further analyzed to determine eye phenotype in heterozygotes with *B* and to determine viability of the exceptional strand relative to stock X-chromosomes. Each such exception was also tested to determine its effect on crossing over in the *f-odsy* or *f-fu* intervals. Finally, preparations of salivary gland chromosomes of progeny of exceptional individuals were studied to determine the cytological basis, if apparent, for the change. Parental stocks were also analyzed cytologically to verify their constitutions.

Results.—Table 1 gives a summary of exceptional male offspring from hemizygous mothers carrying a *f B fu*, intact X-chromosome, and a deficient (lethal) homologue designated $Df(1)B^{263-20}$. According to Sutton[10] this deficiency, obtained by Demerec[11] from an irradiated bar male, includes the *f* locus, all of the left 16A section, and probably all but band $16A_7$ of the right section. Among 69,980 progeny males scored, there were 11 $f B^+ fu^+$ recombinant exceptions, and four $f B^+ fu$ nonrecombinants. Analyses of three of the latter indicate that they are genetically normal and that in each case the change from *B* to B^+ was associated with loss of a single 16A section of the bar duplication. The fourth case was associated with greatly reduced viability.

TABLE 1

NONRECOMBINANT EXCEPTIONAL MALE OFFSPRING* FROM THE MATING: $f B fu/Df263\text{-}20$ ♀ × $f^+ B^+ fu^+$ ♂†

	Analysis of Nonrecombinant Exception				
Nonrecombinant exception	Strand constitution	Viability	Phenotype of heterozygote with *B*	Recombination in *f-fu* region	Salivary chromosome analysis
D-8	$f B^+ fu$	normal	half-bar	normal	one 16A member
D-9	$f B^+ fu$	normal	half-bar	normal	one 16A member
D-10	$f B^+ fu$	normal	half-bar	normal	one 16A member
D-12	$f B^+ fu$	low (stock lost)	...	...	...

* *BB* exceptions not scored. In addition to the nonrecombinant exceptions, there were eleven B^+ recombinants, all $f B^+ fu^+$ in constitution. One other presumptive $f B^+ fu^+$ exception died without mating.
† Total males scored: 69,980.

Exceptional B^+ individuals were also sought among the offspring of *f B odsy/ClB* inversion heterozygotes (Table 2). The *ClB* inversion chromosome carries the bar duplication and the wild-type alleles of *f* and *odsy* in the inverted section, and is lethal in males. Both male and female progeny were searched for B^+ exceptions. As expected, there were no wild-type exceptions that were recombinant for the *f* and *odsy* markers. However, five nonrecombinant B^+ exceptions were obtained. One of these, I-5, involves the parental *Cl"B"* strand, but it has not been determined whether this exceptional *ClB*$^+$ strand carries one or more 16A members.

The other four B^+ exceptions carry the *f* and *odsy* markers of the noninverted parental strand. Two of these cases, I-3 and I-4, are cytologically and genetically aberrant. The remaining two nonrecombinant exceptions, I-1 and I-2, appear normal in all respects. Analyses of salivary chromosomes indicate that the phenotypic change from bar to normal in these cases is associated with loss of a single 16A member.

A search was made for exceptional double-bar individuals among a portion of the male offspring of the mating indicated in Table 2, but none was found.

Table 3 summarizes information on exceptional strands among the progeny of *f B odsy/f*$^+$ *B odsy*$^+$ females mated with f^+B^+ *odsy*$^+$ males. These females were

TABLE 2

NONRECOMBINANT EXCEPTIONAL OFFSPRING* FROM THE MATING:
f B odsy/ClB ♀ × *f*$^+$ *B*$^+$ *odsy*$^+$ ♂

	Analysis of Nonrecombinant Exception				
Nonrecombinant exception	Strand constitution	Viability	Phenotype of heterozygote with *B*	Crossing over in *f-odsy* interval	Salivary chromosome analysis
I-1 ♀	*f B*$^+$ *odsy*	normal	half-bar	normal	one 16A member
I-2 ♂	*f B*$^+$ *odsy*	normal	half-bar	normal	one 16A member
I-3 ♀	*f B*$^+$ *odsy*	lethal	half-bar	reduced	deficiency
I-4 ♀	*f B*$^+$ *od*†	reduced	bar	reduced	new inversion
I-5 ♀	*ClB:* *f*$^+$ *B*$^+$ *odsy*$^+$	"lethal"	half-bar	none	*Cl "B"* inversion

* Total progeny: 42,892 ♀♀; 20,155 ♂♂; there were no crossover exceptions.
† Phenotype is *sy*$^+$; since the proximal breakpoint is close to *odsy* locus, loss of recessive phenotype is probably due to position effect.

TABLE 3

NONRECOMBINANT EXCEPTIONAL OFFSPRING* FROM THE MATING:
f B odsy/f$^+$ *B odsy*$^+$ ♀ × *f*$^+$ *B*$^+$ *odsy*$^+$ ♂
(Progeny totals: 29,414 ♀♀; 27,229 ♂♂)†

	Analysis of Nonrecombinant Exception				
Nonrecombinant exception	Strand constitution	Viability	Phenotype of heterozygote with *B*	Crossing over in *f-odsy* interval	Salivary chromosome analysis
N-1 ♀	*f*$^+$ *B*$^+$ *odsy*$^+$	lethal	bar	reduced	deficiency
N-13 ♀	*f B*$^+$ *odsy*	normal	half-bar	normal	one 16A member
N-14 ♀	*f*$^+$ *B*$^+$ *odsy*$^+$	normal	...	...	...
N-16A ♀	*f B*$^+$ *odsy*	reduced	half-bar	reduced	one 16A member; no detectable aberration
N-16B ♀	*f*$^+$ *B*$^+$ *odsy*$^+$	lethal	bar	reduced	deficiency
N-22 ♀	*f*$^+$ *B*$^+$ *odsy*$^+$	normal	...	...	...
N-24 ♀	*f B*$^+$ *odsy*	lethal	half-bar	reduced	deficiency
N-30 ♂	*f B*$^+$ *odsy*	normal	half-bar	normal	one 16A member
N-31 ♂	*f*$^+$ *B*$^+$ *odsy*$^+$	normal	half-bar	normal	one 16A member
NBB-2 ♂	*f*$^+$ *BB odsy*$^+$	normal	intermediate	normal	three 16A members
NBB-4 ♂	*f*$^+$ *BB odsy*$^+$	normal	intermediate	normal	three 16 A members
NBB-8 ♂	*f*$^+$ *BB odsy*$^+$	reduced	intermediate	reduced	long duplication

* In addition to the nonrecombinant exceptions, there were the following crossover exceptions: 18 *f B*$^+$ *odsy*$^+$ 14 *f*$^+$ *B*$^+$ *odsy*, 3 *f BB odsy*$^+$, and 3 *f*$^+$ *BB odsy*. Two presumptive B^+ exceptions died without mating.
† Double-bar exceptions scored among sons only.

full siblings of the *ClB* hemizygous parents of Table 2. Over 56,000 offspring were searched for exceptions, both sons and daughters for changes from bar to normal phenotype, but sons only for changes from bar to double-bar. In addition to two cases that died without mating, there were 32 crossover B^+ exceptions and nine nonrecombinant B^+ exceptions. Two of the latter, N-14 and N-22, identified in female offspring, exhibited normal viability, but were discarded when it developed that they had the same strand constitution as the male parent, thus rendering their analysis difficult and uncertain. Four other nonrecombinant B^+ exceptions, namely, N-1, N-16A, N-16B, and N-24, were found to be aberrant. The other three B^+ nonrecombinant exceptions, N-13, N-30, and N-31, are genetically and cytologically normal. Like I-1, I-2, D-8, D-9, and D-10 of Tables 1 and 2, their change from bar to normal phenotype was associated with loss of a single 16A member of the bar duplication.

Nine changes from bar to double-bar were identified among male progeny in this experiment. Six of these were recombinant for the *f* and *odsy* markers. The other three (Table 3) represent nonrecombinant *BB* exceptions. NBB-8 exhibits striking genetic abnormalities and is found cytologically to consist of a tandem, serial duplication of a segment of the X-chromosome extending from 16A into 17E. However, NBB-2 and NBB-4 show normal genetic behavior, and salivary gland preparations confirm the presence of three 16A members (triplication) expected of double-bar. It may be recalled that the model for intrachromosomal exchange predicts the occurrence of triplication (double-bar) as well as normal (wild-type) strands from the duplication, without marker recombination.

There was one other case of a nonrecombinant change from bar to double-bar. This exception, RBB-1, has the strand constitution f^+ *BB* $odsy^+$ and occurred as an exceptional male among the progeny of a *f* B^+ *odsy*/f^+ *B* $odsy^+$ female. RBB-1 shows no genetic abnormalities and carries a triplication for the 16A region in its salivary gland X-chromosome. Since it occurred in routine recombination studies, the *f B odsy* male parent did not carry autosomal contamination markers. All things considered, it is extremely unlikely that RBB-1 is a contamination, but if so, it represents the only case in these studies in which a presumptive exception traces to illegitimate parentage.

Studies were undertaken to determine whether the double-bar triplication also yields nonrecombinant, exceptional derivatives. Table 4 summarizes pertinent information on exceptional offspring of appropriately marked females that were homozygous *BB*, or carried *BB* in one chromosome and either *B* or B^+ in the other. These matings produced 62 crossover exceptions and eight exceptions that were nonrecombinant for the markers. Among the latter are two cases of a change from double-bar to normal. One of these, since it was isolated in a female and had the same strand constitution as the father, was not analyzed further. For the other, NBB-1 Rev 19, genetic and cytological evidence indicates that the phenotypic change was associated with a loss of two 16A members of the triplication. In addition, there were five instances of a nonrecombinant change from double-bar to bar phenotype. Genetic and cytological analyses indicate that these strands are not aberrant and, since each carries two 16A members, that the exceptional event was associated with a loss of a single 16A member of the triplication. The case designated NBB-1 Rev 6 is anomalous and illustrates the importance of cytological

TABLE 4

NONRECOMBINANT EXCEPTIONAL OFFSPRING* FROM MARKED *BB/BB*, *BB/B*, AND *BB/B*$^+$ ♀♀† MATED WITH *f*$^+$ *B*$^+$ *odsy*$^+$ ♂♂

(Total strands tested: *BB* 22,606 in ♀♀, 27,903 in ♂♂; *B* 3,906 in ♂♂)

		Analysis of Nonrecombinant Exception		
Female parent	Nonrecombinant exception	Strand constitution	Viability and crossing over in *f-odsy* interval	Salivary chromosome analysis
f BB odsy/f$^+$ *BB odsy*$^+$	NBB-2 Rev 21 ♀	*f*$^+$ *B*$^+$ *odsy*$^+$	discarded	...
f BB odsy$^+$*/f*$^+$ *BB odsy*	NBB-1 Rev 2 ♂	*f*$^+$ *B odsy*	normal	two 16A members
	NBB-1 Rev 3 ♀	*f B odsy*$^+$	normal	two 16A members
	NBB-1 Rev 9 ♂	*f B odsy*$^+$	normal	two 16A members
	NBB-1 Rev 19 ♀	*f*$^+$ *B*$^+$ *odsy*	normal	one 16A member
f BB odsy/f$^+$ *B odsy*$^+$	SBB-1 Rev 1 ♂	*f B* odsy	normal	two 16A members
f$^+$ *BB odsy/f B odsy*$^+$	NBB-1 Rev 6 ♂	*f B*$^+$ odsy$^+$	normal	two 16A members (unchanged)
BB odsy/f$^+$ *B*$^+$ *odsy*$^+$	SBB-2 Rev 5 ♂	*f B odsy*	normal	two 16A members

* In addition to the 8 nonrecombinant exceptions treated in the table, there were 62 crossover exceptions distributed as follows: 10 *B*$^+$ and 31 *B* from *BB/BB* homozygotes; 4 *B*$^+$ from *BB/B* heterozygotes; and 22 *B* from *BB/B*$^+$ heterozygotes. Three presumptive exceptions died without mating.

† All offspring of *BB/BB* mothers were searched for *B* and *B*$^+$ exceptions; all offspring of *BB/B*$^+$ mothers were searched for *B* exceptions only; but only male offspring of *BB/B* females were searched for exceptions.

inquiry as an adjunct to genetic analysis in this type of investigation. The exceptional strand carries markers indicating that it came from the B-carrying chromosome of the *BB/B* parent. However, salivary gland preparations indicate that this strand, which exhibits normal genetic behavior and produces a normal eye phenotype, still carries the bar duplication.

Three of the double-bar to bar revertants of Table 4 were crossed to produce marked heterozygotes with a stock *B* allele, and 21,800 of their offspring were searched for exceptions. In addition to 14 crossover *B*$^+$, and five crossover *BB* exceptions, three noncrossover exceptions were found. One of these, designated FBB Rev 1 Case 2, produces a normal eye phenotype and, since it carries a *f*$^+$ *B*$^+$ *odsy*$^+$ strand, evidently originated from the stock *f*$^+$ *B odsy*$^+$ strand of the mother. This derivative is genetically normal and carries one 16A member. Of the other two, one was identified as a mutation from bar to infrabar, not unlike that obtained as a patroclinous male by Sturtevant.[9] From cytological studies it is apparent that, like the original infrabar, it retains the two 16A members of the bar duplication from which it came. The other exception, designated C-lethal, occurred among the offspring of a *f B odsy*$^+$*/f*$^+$ *B odsy* mother and was identified as having wild-type effect. On the basis of its *f B*$^+$ *odsy* strand constitution it appears to be a crossover. However, it is lethal in males and reduces, but does not eliminate, crossing over in the *f-odsy* segment. The C-lethal exception, it turns out, is deficient for a segment of the X-chromosome extending from 15F into 16E, including the *f* locus and both 16A members of bar, but not the *odsy* locus. Evidently, this aberration occurred in the *f*$^+$ *B odsy* parental chromosome and, since the *f*$^+$ allele was included in the deficient segment, it appeared as a recombinant.

One other exception deserves mention here. It occurred as a single wild-type patroclinous male among the progeny (*ca.* 16,000) of a homozygous *f B odsy*, attached-X female mated with a *g* (garnet) *f B fu* male, in experiments designed to determine whether intrachromosomal recombination occurs in males. It carries the *g*, *f*, and *fu* markers, has normal viability, and gives normal crossing over in the *f-fu* interval. However, in heterozygotes with *B* this derivative gives a typical bar phenotype, and cytological analysis indicates that it retains both 16A members. Apparently it represents another case of mutation.

Discussion.—It is interesting to note that Sturtevant,[9] in his classical work on bar, recorded a single case of nonrecombinant reversion from *B* to B^+, though he considered its status as a valid case doubtful. Also, Braver,[12] in an experiment testing the effect of a nearby inversion on unequal crossing over at the bar locus, obtained (personal communication) a nonrecombinant wild-type revertant from a homozygous bar parent.

In the studies reported here, 29 bar-locus exceptions that were nonrecombinant for marker loci were given detailed genetic and cytological analyses. Of these, eight are associated with aberrations, and three others represent mutations unaccompanied by cytological changes. The remaining 18 cases are of the type expected from intrachromosomal recombination; they exhibit normal genetic behavior, and in each case cytological analysis indicates a gain or loss of 16A members corresponding to the changed phenotype. These include nine changes from *B* to B^+ (loss of one member), three from *B* to *BB* (gain of one member), five from *BB* to *B* (loss of one member), and one from *BB* to B^+ (loss of two members). The frequency of the nonrecombinant *B* to B^+ event (based on 9 cases among 215,376 gametes) appears to be about 1 per 24,000, whereas crossover exceptions of the same type, occurring at the rate of 1 per 1700 gametes (based on 46/78,433), are about 14 times as frequent.

Although the exceptional, nonrecombinant derivatives reported here are expected on the model of intrachromosomal exchange illustrated in Figure 1, the alternative that these exceptions may result from multiple (double) exchanges within the marked segment must be considered. This scheme requires a primary exchange between obliquely synapsed 16A members of the duplication to account for the exceptional bar phenotype, as well as a coincidental exchange involving the same strand and occurring within the marked segment on one or the other side of the bar locus, to produce an apparent noncrossover exception.

If exchange events here are governed exclusively by a mechanism of restrictive chromosomal interference, such double exchanges are not expected since *f* and *odsy* define a segment of only 2.5 map units, which is well beyond the threshold for complete interference. Even assuming that interference is absent, the nonrecombinant exceptions are too frequent, relative to the crossovers, to be explained as doubles, since only 2.5 per cent of strands involved in a primary event at the bar locus are expected to encounter a coincidental exchange between the markers.

In view of the suggestion by Pritchard[13] that multiple exchanges in Aspergillus may take place in localized regions of pairing, and of evidence in Drosophila[14] that is held to support this idea, it is appropriate to consider whether such a mechanism might account for the nonrecombinant bar exceptions. While some exceptions of this type may have such an origin, the evidence and considerations presented below suggest that most, if not all, of them arise intrachromosomally.

Since, on the multiple-exchange hypothesis, the exceptional derivative is dependent on an intrabar primary exchange, an aberration that reduces the frequency of crossover exceptions should occasion a corresponding reduction in the frequency of nonrecombinant (double-exchange) exceptions. Thus, it may be calculated that the deficient homologue of the *B*/*Df*263-20 hemizygote results in a sixfold reduction in B^+ crossover exceptions as compared with the *B*/*B* parent. But the frequency of nonrecombinant B^+ exceptions among the progeny of the *B*/*Df*263-20

hemizygote (3/69,980) is not correspondingly reduced; in fact it approximates that for nonrecombinant exceptions from the nondeficient B/B source (6/145,396).

Another test of the multiple-exchange hypothesis is provided by the B/B^+ heterozygote, for example $f^+ B^+ fu^+/f B fu$. Here $f^+ B fu^+$ strands may conceivably arise as double crossovers but are not expected from intrachromosomal exchange. Among 13,192 gametes from marked B^+/B and B^+/BB heterozygotes, Sturtevant[9] identified six exceptional and 342 nonexceptional single crossovers within the marked segment, but the critical type expected from double exchange was not represented among the progeny. We found no such double crossovers among 25,000 offspring of marked B^+/B heterozygotes, and conclude therefore that multiple exchanges within the *f-fu* segment are rare or nonexistent.

Finally, on the multiple-exchange hypothesis it is anticipated that B/Df hemizygotes, in which one homologue is deficient for all 16A chromatin, and in which therefore the opportunity for interhomologue exchange in the bar region is removed, would yield no nonrecombinant exceptional offspring. To this end we have employed the deficiency designated C-lethal which arose spontaneously in our experiments and which, as already noted, is deficient for a segment extending from 15F into 16E and including the *f* locus, both 16A members, but not *odsy*. Several exceptional individuals have been identified among the offspring of f^+B *odsy*$^+/Df$C-lethal *odsy* hemizygotes, among them two nonrecombinant B^+ revertants designated C-1 and C-2. Both show normal genetic behavior, and cytological analysis of these cases reveals that the change from B to B^+ in each was associated with loss of a single 16A member of the bar duplication. These findings indicate that nonrecombinant exceptions may occur by a mechanism that does not require participation of the homologue; in short, they support the model for intrachromosomal exchange illustrated in Figure 1.

If this mechanism has general significance, it must be taken into account in interpreting instances of anomalous recombination or segregation, such as aberrant tetrads and the so-called conversion phenomena. In fact, the senior author, in a study[15] employing the NBB-8 duplication, has shown that apparent multiple exchanges within a segment of less than three map units are entirely accountable in terms of single exchanges within the serial duplication. It is conceivable that many of the mutants employed in recombination experiments, especially the induced ones, owe their mutant phenotypes to position effects associated with duplications. If so, reversion would accompany removal of the position effect through loss of a duplication member, as either an intrachromosomal or an interhomologue event.

Reddish-alpha, in *Drosophila virilis*, investigated by Demerec,[16, 17] is a case in point. The behavior of this mutable allele of *y* (yellow) was anomalous in that reversions to wild type were, more often than anticipated, associated with crossing over between *y* and the closely linked *sc* (scute) locus. We think it likely that reddish was a serial duplication in which one of the breaks occurred near the y^+ allele, giving a position-effect reddish phenotype. Reversions to wild type are thus interpretable as losses of one member of the duplication occurring either through intrachromosomal exchange (nonrecombinant) or through legitimate exchanges between homologues (crossover), much as alpha is isolated from the alpha:beta complex in maize.

Intrachromosomal exchange may be significant for the evolution of genetic

systems. We note that the mechanism calls for gain, as well as loss, of duplication members, and that several instances of nonrecombinant changes from bar to double-bar were identified in this study. It may be suggested that intrachromosomal exchange provides a model for the highly specified addition of genetic material, and that it may be of special significance for organisms in which interhomologue events may not be available for such additions to the genome. It is particularly inviting to consider that adjacent, functional units governing related processes may have such an origin.

Summary.—Genetic and cytological analyses of 29 bar locus exceptions that were nonrecombinant for marker loci reveal that 18 of these changes were associated with discrete loss or gain of 16A members of the salivary chromosome. In addition two such exceptions were identified among progeny of hemizygotes carrying an X-chromosome homologue deficient for 16A chromatin. From this and other evidence it appears that these nonrecombinant exceptions are not the result of multiple exchanges (negative interference). Rather, they support the model for intrachromosomal exchange.

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*CYTOCHEMICAL STUDIES OF NUCLEIC ACIDS AND PROTEINS IN ERYTHROCYTIC DEVELOPMENT**

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The process of erythrocytic production offers an excellent system for the study of the relationship between nucleic acids and proteins during differentiation. All stages of development can be obtained in a single given tissue at a given time, and hemoglobin can be used as an indication of the specialization attained. Reticulocytes have provided a relatively homogeneous system for biochemical studies on the relationship of hemoglobin synthesis to reticulocyte RNA.[1-11] While such investigations are obviously of considerable experimental value, they do not reveal the modifications which had occurred prior to the reticulocyte stage and which undoubtedly influenced the metabolic behavior of the reticulocyte. Furthermore, biochemical studies of blood cell formation are limited by the marked heterogeneity in cell populations of various hematopoietic organs and the considerable difficulties in achieving a satisfactory separation of the many cell types.

On the other hand, cytochemical studies can be advantageously employed, since the need for separation of the various cell types is eliminated and cell identification within a given sample rendered possible through direct examination. This investigation examines the sequence of changes in nucleic acid and hemoglobin amounts during the process of development by means of microphotometry and radioautography.

Materials and Methods.—Livers were obtained from 19- or 23-day-old fetal rabbits after administration of Nembutal to the mother. Slides for microphotometric measurements were prepared by touching the freshly cut surface of the liver to a glass slide. These touch preparations or imprints were immersed for 1–2 min in absolute methanol or 10% formalin (pH 7.0) buffered in Sorensen phosphate buffer and subsequently air-dried. For RNA determination, the preparations were stained with Azure B,[13] and mounted in a mounting medium whose refractive index matched that of the cells (see Table 1). For measurement of total protein, Azure B was removed in water adjusted to pH 2.0 with 0.1 *N* HCl and slides restained in 0.001% fast green (FCF) at pH 2.0.[14] The preparations were remounted in a matching refractive index medium and total protein measured in the same cells used for RNA determinations. Hemoglobin measurements were made in the Soret band (420 mμ) on imprints which were lightly stained in Azure B to facilitate recognition and identification of the various erythropoietic cells. At 420 mμ, the light Azure stain gave unmeasurably low extinctions and therefore did not interfere with hemoglobin absorption. Table 1 summarizes the techniques employed for each cellular component. The microphotometric instrumentation and details of the methods used have been previously described.[15] In calculating amounts of bound dye or hemoglobin, the formula M = EA was used where M = amount of bound dye or hemoglobin, E = extinction, and A = area as determined on camera lucida drawings with the polar planimeter. The amount of DNA (Feulgen) per nucleus was calculated by the two-wavelength method.[15, 16]

To justify the use of the formula M = EA on imprints, it was necessary to demonstrate the uniform thickness of individual cells upon the slide. This was performed by obtaining extinctions of variable-sized concentric plugs starting at the center of the cell and progressively increasing plug size until almost the entire cell was included in the field. If the cell was of uniform thickness throughout, one would expect the extinctions obtained at all plug sizes to be equal. Since the extinctions showed no significant variation, it was concluded that the cells in imprints were uniformly flattened disks (see Korson[17]).

TABLE 1

SUMMARY OF TECHNICAL PROCEDURES

Substance measured	Stain	Fixative	Wavelength in mμ	Microphotometric method	Refractive index of mounting medium (n)
1. RNA	Azure B	methanol	530	plug[15]	1.564
2. RNA	Azure B	formalin	530	plug	1.564
3. Total protein	Fast green	methanol	536	plug	1.564
4. Hemoglobin	Natural pigment	methanol	420	plug	1.560
5. Hemoglobin	Natural pigment	formalin	420	plug	1.560
6. DNA	Feulgen reaction	formalin	480; 501	two-wavelength[15, 16]	1.564

Radioautographic analysis of RNA synthesis was performed on 19-day fetal rabbits. The mother was anesthetized with intravenous Nembutal and laparotomy performed. Two hundred μC of tritiated cytidine (specific activity 1.25 C/mM) was injected into the placenta by passing a fine bore needle through the uterine wall. At the end of 6 hr, the mother was sacrificed, and the livers excised from the injected fetuses. Tissues were fixed in cold (0–5°) 10% buffered formalin (pH 7.0) or Zenker-formol for 1 hr and embedded in paraffin. After extractions to ensure specificity of incorporated label, slides were coated with stripping film (Kodak AR-10) and developed after a suitable exposure period. Grain counts were made over the nucleolus, nucleus, and cytoplasm after staining in hematoxylin-eosin-azure[18] or Azure B, and the count expressed in number of grains per square micron. The correction for background was included in the data.

Identification of the various erythropoietic cells was based on the nuclear morphology and cytoplasmic tinctorial properties. In addition, it was found that nuclear area could be arbitrarily used in cellular identification and, for this reason, values were plotted against this parameter. The sequence of erythropoietic cells was considered as follows: hemocytoblast→ proerythroblast→ basophilic erythroblast→ polychromatophilic erythroblast→ normoblast→ reticulocyte→ erythrocyte.[19]

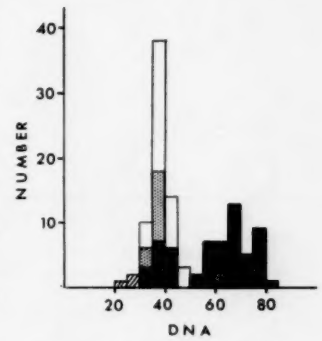

FIG. 1.—Amount of DNA per erythroblast nucleus. Open bars represent late normoblast nuclei; stippled bars represent nuclei in the process of extrusion; cross-hatched bars represent extruded nuclei; solid bars represent a mixed population of erythroblast nuclei exclusive of late normoblast nuclei. Photometric units in abscissa are calculated by the two-wavelength method.[16]

Results.—(1) *DNA:* Microphotometric measurements of DNA in a randomly selected population that included all stages in the developmental sequence result in the composite or "pooled" data depicted in Figure 1. The data show a bimodal distribution into 2C and 4C classes, as well as a number of interclass values characteristic of a proliferating system of cells. In a randomly selected population of late normoblasts, however, all fell into the 2C class, suggesting the cessation of DNA synthesis and mitotic activity at this stage. A similar distribution of DNA values was observed in nuclei which appeared to be in the process of extrusion. Only after extrusion was completed was a depression in DNA amount per nucleus indicated.

(2) *RNA:* (a) *Microphotometry:* The amount of cytoplasmic RNA per cell was initially high in the hemocytoblast or stem cell (Fig. 2). In successive stages of development, cytoplasmic RNA exhibited a constant and gradual decrease throughout the process of maturation (Fig. 2). No detectable Azure B dye was bound by mature erythrocytes; the slight absorption (E = 0.041) at 530 mμ was shown by curve analysis to be due to porphyrins. The decline in RNA amount per cell was

TABLE 2

INCORPORATION OF H^3 CYTIDINE (200 μC) PRESENTED TO NINETEEN-DAY RABBIT FETUS FOR SIX HR

Stage	Grains/μ^2*			No. cells
	Cytoplasm	Nucleus	Nucleolus	
HCB-PrE	0.17	0.42	1.42	40
BE	0.14	0.35	...†	13
PE	0.067	0.061	0	21
Subsequent stages	0	0	0	

* After correction for background.
† Value not significant due to small size of nucleoli in cells sampled.

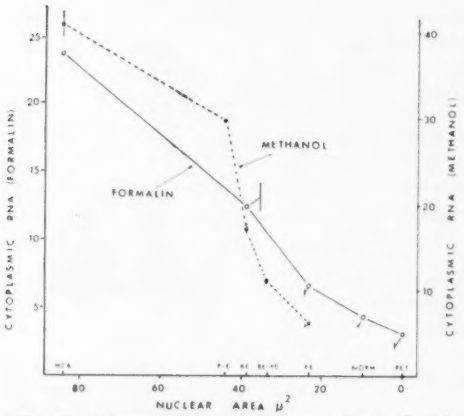

FIG. 2.—Means and standard errors of cytoplasmic RNA amounts throughout blood cell development. HCB = hemocytoblast; PrE = proerythroblast; BE = basophilic erythroblast; PE = polychromatophilic erythroblast; NORM = normoblast; RET = reticulocyte. Photometric units in ordinate are calculated from extinction X area in square microns.

accompanied by a progressive decrease in RNA concentration (following a slight increase in the basophilic erythroblast) and also by a substantial decrease in cell size measured in terms of cytoplasmic area (see below).

(b) *Radioautography:* With presentation of H^3 cytidine for 6 hr, marked incorporation into RNA was observed in the hemocytoblast, proerythroblast, and basophilic erythroblast (Table 2). All later stages exhibited no incorporation of labeled precursor. In the labeled stages, the highest activity was found in the nucleolus, followed successively by the nuclear and cytoplasmic compartments. In the polychromatophilic erythroblast, a light label was found equally distributed over the nucleus and cytoplasm. This label could result from cells that had differentiated during the 6 hr presentation time. This explanation is supported by the fact that less than 20 per cent of 122 cells were labeled and that the label did not show a nuclear concentration. Therefore, it was concluded that only the earliest stages exhibited RNA synthesis and that, if subsequent stages were capable of RNA synthesis, there was a substantial decrease in their ability to incorporate the precursor.

(3) *Protein:* Cytoplasmic total protein, measured in the same cells used for RNA determinations, decreased between the hemocytoblast and polychromatophilic erythroblast and then increased sharply to the reticulocytic stage (Fig. 3). The initial decline in cytoplasmic protein observed in the earlier phases of development accompanied a substantial decrease in cytoplasmic area. The mean area of the hemocytoblast was 59 μ^2 compared to a value of 14 μ^2 in the polychromatophilic erythroblast, while extinctions remained constant or were slightly increased during the same period. The increase observed in later phases was attributed to intensive synthesis of hemoglobin and is in agreement with microchemical and ultraviolet absorption measurements made in bone marrow erythroblasts by Thorell.[20] Soret band measurements showed the sharpest increase in total amounts of porphyrin per

cell occurring during the later developmental stages (Fig. 4). Because of the low extinctions obtained at 420 mμ prior to the polychromatophilic erythroblast, hemoglobin amounts for these stages may be overestimated by nonspecific light loss. The nuclear protein measurements showed a decline throughout the erythropoietic process which was accompanied by a decrease in nuclear area (Fig. 3). No measurements were made of nuclei which had been extruded.

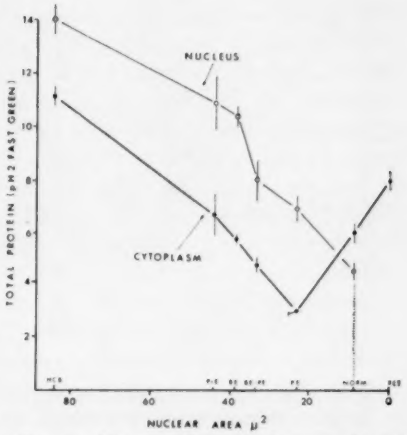

FIG. 3.—Means and standard errors of total protein amounts in the nucleus and cytoplasm at different stages of blood cell development. The curve of cytoplasmic changes represents the pooled results of two experiments. Photometric units in ordinate are calculated from extinction X area in square microns.

Discussion.—The results (Fig. 5) suggest that the metabolically stable RNA complex demonstrated in reticulocytes[11, 21–23] is established at the time of differentiation into the erythrocytic line. This interpretation is supported by several of our observations which can be considered in two categories: (1) the lack of cytidine incorporation into late erythroblast RNA, and (2) the inverse relationship between RNA and hemoglobin.

(1) *Lack of cytidine incorporation into erythroblast RNA:* The absence of H^3-cytidine incorporation into the RNA of erythroid cells subsequent to the basophilic erythroblast stage presumably reflects the lack of ribosomal RNA synthesis in erythroblasts which, in turn, results in a gradual loss of cytoplasmic RNA. Although our data do not preclude the possibility that an extractable soluble RNA fraction is in a state of turnover, this seems unlikely since biochemical results have been reported in which the rate of RNA turnover in reticulocytes has been considered too low to represent formation of a messenger RNA.[11, 21–23] Furthermore, Marks *et al.* pointed out that the low incorporation of C^{14}-guanine in whole cell preparations could be due to leukocytic contamination or to terminal addition of the label to reticulocyte soluble RNA.[11]

(2) *Inverse relationship between RNA and hemoglobin:* From Figures 3 and 4, it is evident that the most active period of hemoglobin synthesis occurs in the later stages when cytoplasmic RNA is relatively low (see also review by Errera[24]). Ackerman has reported that histochemical demonstration of protein-bound sulf-

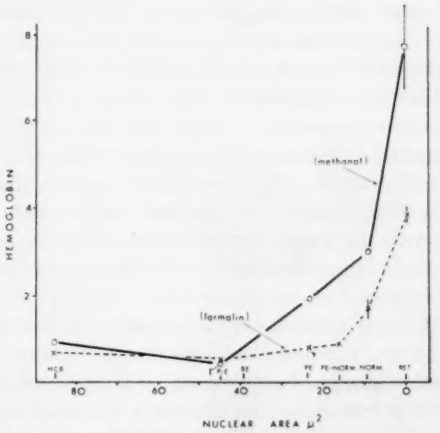

FIG. 4.—Means and standard errors of hemoglobin amounts during erythrocytic production. Photometric units in ordinate are calculated from extinction X area in square microns.

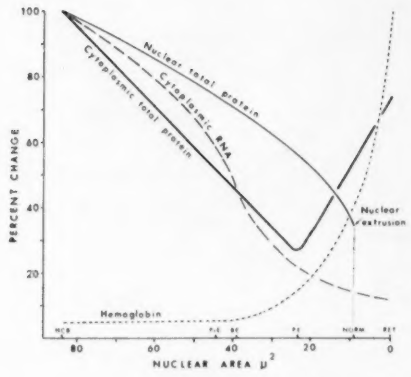

FIG. 5.—A summary of the data showing the cytochemical changes involved in blood cell production.

hydryls and histidine presumed to be associated with the globin moiety was most intense during this same period.[25] Although it can be assumed that some globin is present in earlier stages, on the basis of Ackerman's results and electron microscopic observations,[12] its presence is masked by the loss of proteins other than hemoglobin which constitute the bulk of cellular proteins during earlier periods.

The "delayed" appearance of hemoglobin (relative to RNA) is in direct contrast with many cellular systems where protein synthesis is usually accompanied or preceded by a period of intense RNA synthesis. The delay is explicable if the presence of a stable informational complex is postulated, a complex which is probably integrated with a small potentially "active" ribosomal fraction. In this way, a decrease in cytoplasmic RNA represented by the loss of an "inactive" ribosomal fraction is possible without hindering the cell's ability to synthesize hemoglobin. Indeed, electron microscopic examination of forming erythroid cells indicated the presence of both single ribosomes and ribosomal clusters.[12] Recent studies of hemoglobin synthesis in rabbit reticulocytes utilizing incorporation of labeled amino acids into ribosomes of intact cells and cell-free preparations indicated that less than 20 per cent of the total ribosomes were associated with newly-formed peptides.[11] In a similar system, Warner *et al.* found the "active" ribosomal fraction chiefly associated with a pentameric ribosomal complex which sedimented at 170S.[10] Single ribosomes with a sedimentation rate at 76S were demonstrated to be "inactive" in the incorporation of labeled amino acids.

The nuclear alterations observed during erythropoiesis are the result mainly of a decrease in total protein and probably water, but do not arise from a loss of DNA, as has been suggested.[17] It is evident from Figure 1 that the entire population of erythroid elements except the late normoblast exhibits 2C and 4C classes and several interclass values characteristic of a population undergoing DNA synthesis and mitosis. All normoblasts contain the diploid DNA level typical of a nondividing tissue. If, however, one considers the mean of all DNA values at each particular stage, the bimodal distribution would be obliterated and a gradation of mean values obtained in a fashion suggestive of a DNA loss, as reported by Korson.[17]

Several investigators have concluded that the normoblast nuclei disappear through intracellular karyolysis[17,26] rather than by extrusion.[31] However, apparent nuclear expulsion has been frequently observed by us both in the light and electron microscope. Furthermore, a 2C amount of DNA was found in extruding nuclei; lower values were found only in extruded nuclei.

It would seem, then, that the primary function of erythroblast nuclei during the greater part of the erythropoietic process is to provide a means for proliferation and that, after the basophilic erythroblast stage, the nucleus plays a minor role, if any,

in further differentiation of the system. Support for this conclusion can be drawn from several observations. Electron microscopic examination of hemocytoblasts and proerythroblasts revealed the presence of several possible pathways of nucleocytoplasmic exchange which were gradually lost in later stages.[12] In addition, it has been shown that the nucleus may be expelled in the polychromatophilic erythroblast during the normal course of development as well as under anemic stress.[27] Marrow cells *in vitro* exhibit a tendency to extrude their nuclei prior to active hemoglobin synthesis, but incorporation of heme precursors continues to occur.[28] Finally, the occurrence of hemoglobin synthesis in the nonnucleate reticulocyte is further evidence that nuclear presence is not required for synthesis of hemoglobin.[2-11] In view of these findings, one cannot assume that the presence of hemoglobin described within nuclei of later erythroblasts[12] or the mature nucleated erythrocytes of lower vertebrates[29,30] plays a major functional role in differentiation. As we have pointed out earlier from fine structure studies, its presence in nuclei appears to be a later acquisition, long after the establishment of a definite developmental pattern.

We conclude that differentiation along the erythrocytic lineage results in the establishment of a metabolically stable informational RNA complex in the earliest stages which is retained throughout the remainder of the developmental process. After the establishment of this complex, there is a loss of RNA synthesis, evident from the lack of H^3-cytidine incorporation, and a gradual decrease in cytoplasmic RNA levels. At a point when the RNA content is relatively low, intensive hemoglobin synthesis is initiated, which suggests that the stable informational complex is integrated into a small "active" ribosomal fraction. This "active" fraction must be retained while the larger "inactive" ribosomal population is deleted. After the basophilic erythroblast stage, nuclei continue to divide, thus enabling proliferation of the system, but eventually become mitotically inactive and are expelled from the cell. The role of the nucleus, then, would seem to be twofold: (1) in the earliest stages, the formation of the stable informational complex; and (2) to provide a means of cellular proliferation.

Summary.—Microphotometric measurements of various stages of red blood cell formation indicated a gradual loss of cytoplasmic RNA throughout the process. In later stages both total protein and hemoglobin revealed a concomitant increase. Autoradiographic analysis of RNA synthesis utilizing tritiated cytidine showed no incorporation after the basophilic erythroblast stage. DNA measurements revealed a bimodal distribution into 2C, 4C, and intermediate values in all stages except the late normoblast. The latter contained the 2C amount of DNA, while extruded nuclei revealed a decrease in DNA. It was concluded that: (1) a stable informational RNA complex is formed only in the earliest developmental phases and is retained throughout the process; (2) the loss of RNA in early stages is chiefly limited to an "inactive" ribosomal fraction while the "active" fraction is retained; and (3) the nuclei serve both as the origin of the stable messenger and as a means for proliferation of the system.

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† Postdoctoral Research Fellow of the National Heart Institute, National Institutes of Health.

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*ANALYSIS OF UNLINKED SUPPRESSORS OF AN O° MUTATION IN SALMONELLA**

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The nature of the operator region of an operon in bacteria has been primarily defined in terms of the characteristics of its mutant forms. One type of mutant alteration of an operator, designated O°, results in the nonfunctioning of the associated structural genes, regardless of the conditions of growth.[1] Evidence has been presented which indicates that an operator does not act via a cytoplasmic product. Jacob and Monod have suggested that it acts as a receiver of controlling signals (i.e., the receptor of the repressor) and as the initiating point for the transcription into messenger RNA of the information carried by the structural genes of

the operon. Therefore, an O° mutation might be expected to result from any type of alteration of an operator which blocked the transcription process.

A leucine auxotroph, *leu 500*, has been shown to have resulted from an O° mutation affecting the leucine operon in *Salmonella typhimurium*.[2] In the present paper we present evidence concerning the location and nature of the genetic alterations which act as suppressors of the leucine O° mutation.

Materials and Methods.—The bacteria used were all derivatives of strain LT2 of *S. typhimurium*. A number of mutant strains bearing markers in the tryptophan genes and the cysteine B genes were kindly provided by Dr. M. Demerec. The general procedures for transductions using P22 phage have been described previously.[2] The minimal medium used,[2] unless otherwise specified, contained sodium citrate and glucose, and was supplemented with appropriate amino acids (when needed) in concentrations described by Novick and Maas.[3] Tests for utilization of various sugars were carried out on a citrate-free minimal medium containing the appropriate sugars (0.2%). Difco nutrient broth was used for growing cultures for phage lysates or transductions, except for the strains bearing suppressor mutations. A broth medium consisting of 1.0% Difco tryptone and 0.5% Difco yeast extract was used for these latter strains.

Results.—The O° mutant, *leu 500*, when spread upon minimal agar medium and incubated at 37°C for 72–148 hr, gave rise to colonies of leucine-independent cells. These colonies were readily divided into two classes based upon size. Relatively few were large and during incubation increased in size at approximately the same rate as colonies of wild-type cells. The majority, however, fell into a class of distinctly smaller colonies. These never approached the size of the large colonies regardless of the length of the incubation period. Such "partial revertants" have frequently been found to be the result of linked[4] or unlinked[5] suppressor mutations. Therefore, the strains selected from the small leucine-independent colonies will hereafter be referred to as suppressor strains. For the purpose of this report the strains obtained from large leucine-independent colonies will be presumed to be true revertants, since the resultant phenotypes appear to be that of the wild-type strain LT2.

When aliquots of a nutrient broth culture of the O° mutant, *leu 500*, were spread upon minimal agar medium which was supplemented with all amino acids (except leucine) plus all the normal purine and pyrimidine bases, large and small leucine-independent colonies also appeared. When these colonies were replica plated onto unsupplemented minimal agar medium, approximately 5% of the small colonies were unable to grow. Analysis revealed that the latter colonies were auxotrophic for cysteine or tryptophan, or both cysteine and tryptophan. The following table enumerates the results of a sampling of 1190 leucine-independent colonies appearing upon the leucine-free amino acid medium (10^{10} cells of *leu 500* tested):

Size of colonies	Prototrophs	Cysteine auxotrophs	Tryptophan auxotrophs	Cysteine-tryptophan auxotrophs
Large	197	0	0	0
Small	946	31	11	5

To study this phenomenon a number of leucine-independent colonies of each type were selected and purified by means of single colony isolation procedures. The independent origins of these strains were assured by using 10 different subcul-

tures of *leu 500*, obtained from single colony isolates, and selecting no more than one of each type of suppressor mutant or revertant from each subculture. The leucine-independent strains studied included 10 presumed true revertants (large colonies), 10 prototrophic suppressor mutants (small colonies), 10 cysteine-requiring suppressor mutants, 7 tryptophan-requiring suppressor mutants, and 7 suppressor mutants which required both cysteine and tryptophan.

The involvement of both cysteine and tryptophan immediately focused our attention upon the portion of the Salmonella chromosome containing the cysteine B region and the tryptophan cluster of genes. Demerec and Hartman[6] as well as Clowes[7] have noted that cysteine B and tryptophan markers of Salmonella are very closely linked. Demerec and Hartman reported a frequency of joint transduction for markers in these two regions which varied from 18 to 44%. It should be noted, however, that the leucine operon is not linked by transduction to the cysteine B and tryptophan regions of the chromosome. Evidence obtained from studies of the *Escherichia coli* chromosome by conjugation indicates that the leucine region is quite some distance from the tryptophan region with at least several known genes intervening.[8] Similarity of the order of genes on the *S. typhimurium* and *E. coli* chromosomes[9] indicates that this is also probably true in *S. typhimurium*.

The distribution of the auxotrophic suppressor mutants into cysteine requirers, tryptophan requirers, and cysteine plus tryptophan requirers suggested that the region of the chromosome responsible for the suppression of *leu 500* lay between cysteine B and the tryptophan cluster of genes. The auxotrophic suppressor mutants would then represent three classes of large multisite mutations, probably deletions. One class would have aberrations extending from the suppressor region to the cysteine B genes on one side (strains requiring cysteine for growth). A second class would involve aberrations extending from the suppressor region to the tryptophan genes on the other side (strains requiring tryptophan for growth). The third class would consist of very large multisite mutations extending from cysteine B to the tryptophan genes and involving both, as well as the suppressor region between them. This region of the chromosome will therefore be designated as the *su leu 500* locus.

One additional characteristic of some of the auxotrophic suppressor mutants was noted. When sodium citrate was omitted from the minimal medium, certain of the suppressor mutants were unable to grow despite the presence of sufficient quantities of glucose and the required amino acids. Upon further testing it was found that these same strains were also unable to utilize arabinose, galactose, maltose, mannose, sorbitol, and mannitol. Four of the seven tryptophan-requiring strains and three of the seven strains which required both cysteine and tryptophan showed this carbohydrate negative character. In contrast, all 10 cysteine-requiring suppressor mutants, as well as the 10 prototrophic suppressor mutants and 10 presumed true revertants, were capable of utilizing all of the carbohydrates listed above. This association of the carbohydrate negative characteristic with tryptophan auxotrophy suggested that a locus (or loci) associated with the utilization of these substances was located near the cluster of tryptophan genes and on the side opposite to that occupied by the *cysB* and the *su leu 500* loci. This region of the chromosome identified with carbohydrate utilization will be designated as the *car* locus.

It seemed reasonable to suspect that the strains obtained from the small proto-

trophic colonies resulted from point mutations and perhaps minute deletions within the *su leu 500* locus. As mentioned previously, the large colony prototrophs seemed likely to have resulted from true back mutations at the *leu 500* site within the leucine operator.

Figure 1 is a diagram of our interpretation of the relative positions of the loci

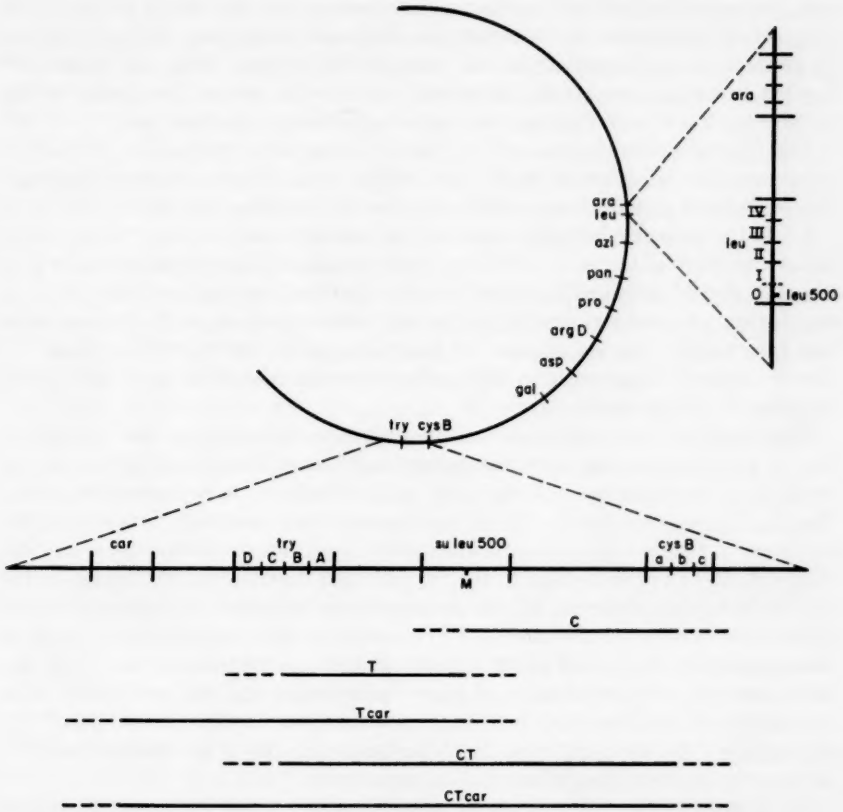

FIG. 1.—Diagram indicating the locations and probable nature of the mutations which suppress the O° mutant, *leu 500*. The suppressor locus is designated as *su leu 500*, and the locus associated with carbohydrate utilization is designated as *car*. The lines below the chromosome segment that contains the *su leu 500* locus represent the approximate extents of the various multisite mutations. *M*—point suppressor mutation (or minute deletion) resulting in prototrophy. *C*—multisite suppressor mutation causing cysteine requirement. *T*—multisite suppressor mutation causing tryptophan requirement. *Tcar*—multisite suppressor mutation causing tryptophan requirement and inability to utilize certain carbohydrates. *CT*—multisite suppressor mutation causing both cysteine and tryptophan requirement. *CTcar*—multisite suppressor mutation causing cysteine plus tryptophan requirement and inability to utilize certain carbohydrates.

involved and of the nature of the suppressors of *leu 500*. The relative positions of the various genes on the semicircular chromosome in the diagram are derived from an unpublished composite map assembled by Taylor, Sanderson, and Demerec, using data from many sources, both published and unpublished. The order of the

cysB genes and the *try* genes, within each cluster, are diagramed here according to the findings of Mizobuchi, Demerec, and Gillespie[10] and of Demerec and Hartman,[6] respectively. Yanofsky and Lennox[11] had found a slightly different order for the *try* genes in *E. coli*. Recently, Balbinder,[12] studying *S. typhimurium*, obtained evidence indicating an order of *try* genes similar to that of *E. coli*, in contrast to the findings of Demerec and Hartman. Utilizing the material described here, we have obtained very preliminary evidence which appears to be consistent with the interpretation of Demerec and Hartman concerning the order of the *try* genes in *S. typhimurium*, as well as evidence for more than one operator.[13] Further investigations of the structural relationships among the genes of the tryptophan cluster and their genetic regulating elements are under way.

The interpretation diagramed in Figure 1 permits a number of predictions concerning the behavior of *su leu 500* strains under various conditions of test. The predictions plus brief summaries of the results of the tests follow.

1. If the prototrophic suppressor strains are the result of point mutations or minute deletions in the *su leu 500* locus, then the genetic basis of their leucine prototrophy should be linked by transduction to *cysB* and *try* markers but not to an *ara* marker. In contrast, the genetic basis of leucine prototrophy in the presumed true revertants of *leu 500* should not show linkage to *cysB* and *try* markers. It should display linkage with an *ara* marker since the arabinose genes are jointly transduced with the leucine operon.[2]

Transductions were performed utilizing all 10 prototrophic *su leu 500* strains and all 10 presumed true revertants as donors. Three strains bearing the *leu 500* mutation in combination with *try*, *cysB*, and *ara* markers were used as recipients. The results were conclusive. In all the transductions involving the prototrophic suppressors, the genetic elements which introduced leucine prototrophy into the recipient showed close linkage to the *try* and *cysB* markers but no linkage to the *ara* marker. As predicted, all the presumed true revertants demonstrated joint transduction of leucine prototrophy and their *ara* marker, but gave no evidence of linkage between their *cysB* or *try* markers and the introduction of *leu*$^+$. In this latter case the joint transduction of leucine prototrophy and the *ara* marker, while consistent with our presumption of true reversions, does not eliminate the possibility of suppressor mutations at sites closely linked to the site of the *leu 500* mutation and capable of restoring complete leucine prototrophy.

2. If many of the prototrophic suppressor colonies result from point mutations in the *su leu 500* locus, then mutagens which specifically induce point mutations should increase the frequency with which such suppressor mutations occur. In contrast, the frequency of occurrence of auxotrophic suppressor mutations, resulting from deletions, should not be increased by such agents.

The mutagenic agent 2-aminopurine was selected to test this prediction. The mechanism proposed by Freese[14] for the induction of mutations by this agent would be expected to cause single site mutations. Demerec[15] presented evidence that 2-aminopurine does not increase the frequency of deletions while sharply increasing the frequency of point mutations.

The effect of a very brief exposure of a culture of *leu 500* to 2-aminopurine was quite in accord with the prediction above. An untreated control sample of the culture produced 90 prototrophic suppressor mutants and 8 auxotrophic suppressor

mutants (3 cysteine requirers, 3 tryptophan requirers, and 2 which required both cysteine and tryptophan). An equivalent sample of the 2-aminopurine-treated culture produced 242 prototrophic suppressor mutants and only 2 auxotrophic suppressor mutants, both tryptophan requirers.

3. If the auxotrophic suppressor strains result from deletions, then no true back mutations should occur. This was readily tested by spreading aliquots of each of the 24 strains upon minimal agar medium supplemented with leucine. Following 96 hr of incubation the plates were examined for colonies. Only one strain, auxotrophic for tryptophan, produced any colonies. Replica plating of these colonies onto unsupplemented minimal medium demonstrated that every tryptophan-independent colony still retained the leucine independence. Thus, these colonies did not represent true reversions since such occurrences would have resulted in a return to the leucine auxotrophy of the original *leu 500* strain. The basis of the tryptophan prototrophy in these colonies is currently being investigated.

The absence of any true reversions is consistent with the interpretation of the auxotrophic suppressor mutations as deletions.

4. Deletions extending into or through the *cysB* and *try* gene clusters should delete these genes in an order consistent with their order on the chromosome. To test this prediction we initially examined the effects of the presumed deletions on the *try* genes, since this cluster of genes was the better known of the two.

For recombination tests four tryptophan auxotrophs were selected as tester strains, each bearing a mutation in a different *try* locus (*tryA*, *tryB*, *tryC*, and *tryD*). Reciprocal transductions were performed between each of these and the 14 auxotrophic suppressor mutants which had a tryptophan requirement. These were then scored for the presence of try^+ recombinants. Ten of these suppressor strains appeared to involve deletions which eliminated all four *try* loci, since no try^+ recombinants were produced from any of the transductions in which they were used. As would be predicted, all seven suppressor strains which showed the carbohydrate negative character were among these ten.

Four of the *su leu 500* strains appeared to result from aberrations extending for various distances into, but not through, the tryptophan cluster of genes, since try^+ recombinants were produced from transductions with some of the tester strains. In every case the results were consistent with the order of the *try* loci presented in Figure 1. For example, two of these suppressed mutants produced no try^+ recombinants from transductions involving the *tryA* strain, but did produce try^+ recombinants from transductions involving the *tryB*, *tryC*, and *tryD* strains. We have concluded that these strains resulted from deletions extending into and perhaps through the *tryA* gene, but ending before reaching the site of the mutation carried by the *tryB* strain. In no case did we find evidence of a suppressor mutation which affected the *try* locus most distant from the *su leu 500* locus without also affecting the *try* loci closer to the *su leu 500* locus. Further details of this analysis will be presented in a later publication.

5. If the auxotrophic suppressor strains resulted from deletions, then transductions should reveal 100% linkage among all the genes located within each deletion. To test this prediction a *car, try, su leu 500, cysB, leu 500* strain was transduced by phage grown on wild type (car^+, try^+, *su leu* 500^+, $cysB^+$, leu^+). Selection was made only for the introduction of the ability to utilize carbohydrates (car^+), i.e.,

the transduction mixture was spread upon minimal agar medium containing glucose but no citrate, and supplemented with cysteine, tryptophan, and leucine. All resulting *car*$^+$ colonies, when tested on appropriate media, were found to be prototrophic for cysteine and tryptophan, and auxotrophic for leucine (*car*$^+$, *try*$^+$, *su leu 500*$^+$, *cysB*$^+$, *leu 500*). Similarly, when the introduction of two of the other characters (*cysB*$^+$ or *try*$^+$) was individually selected for on appropriate media, once again 100% linkage was observed when all four characteristics were examined. The same results were obtained with several other auxotrophic suppressor strains; in these, only the linkage of the *cysB*, *try*, and *su leu 500* loci were examined.

Discussion.—The evidence presented here is consistent with the interpretation of the auxotrophic suppressor mutations as being either deletions, inversions, or some as yet unknown form of aberrations which inactivate relatively large segments of chromosomes with respect to both physiological functioning and recombination. Deletions appear to be the most reasonable explanation for these results. The positive selective value of such deletions in the *leu 500* strain (on minimal medium supplemented with cysteine and tryptophan) provides an excellent opportunity to investigate the mechanisms which cause deletions. Studies of the effects of various agents and growth conditions upon the induction of auxotrophic suppressor mutations are being undertaken.

The occurrence of unlinked suppressor mutations affecting structural gene mutations is quite common. In most instances the effect of such suppression is to overcome the inactivity of a single enzyme. In the case described here, however, the *leu 500* mutant lacks three different enzymes.[2] This suggests that the suppressor mutations probably act through their effect upon the altered leucine operator of strain *leu 500* and not by directly affecting the structural genes or their products. This view is supported by our evidence[13] that the *su leu 500* mutations, when inserted by transductions into the unsuppressed *leu 500* strain, bring about leucine prototrophy, but do not do so when inserted into leucine auxotrophic strains bearing mutations in the structural genes.

It is apparent from the preponderance of prototrophic suppressor colonies in Table 1 that the cysteine and tryptophan auxotrophy are not essential to the suppression of *leu 500*. Furthermore, the induction of suppressor mutations by 2-aminopurine suggests that suppression can be achieved by altering the bases of a single nucleotide pair and that deletions are not an essential characteristic. One suggested mechanism for restoring the activity of structural genes that are associated with an O° mutation is an aberration which puts the structural genes in association with a functioning operator.[16] That this is not the mechanism which functions in the suppression of *leu 500* is indicated by the evidence that single base-pair alterations in the *cysB-try* region of the chromosome will suffice. This is reinforced by our evidence that leucine prototrophy results from the introduction, by transduction, of the deletion type of suppressor mutations into the *cysB-try* region of the chromosome.

The evidence that complete deletion of the *su leu 500* locus (suppressor strains requiring both cysteine and tryptophan) serves to suppress *leu 500* suggests that the suppression results from the inactivation or elimination of a normal cell constituent which is a direct or indirect product of the *su leu 500* locus. Since operators are envisaged as normally responding to the presence of repressors, it seems reasonable

to consider the possibility that the *su leu 500* locus is a regulator gene. Our initial hypothesis pictured the *su leu 500* locus as the leucine regulator gene. The mutation which gave rise to *leu 500* was visualized as an alteration of the leucine operator which permitted the normal leucine repressor substance to act as a superrepressor, thereby creating an O° phenotype. Any mutations which inactivated or eliminated the leucine regulator gene in such a mutant would thus relieve the O° condition, and presumably the leucine operon would then function constitutively.

Burns[17] has made a study of the enzymes concerned with leucine biosynthesis in the various types of suppressed mutants. His results revealed that the level of the enzymes in these strains are regulated by the amount of leucine made available to the cells in the medium, although the levels were always considerably lower than that of the wild-type strain. This regulation of enzyme level by leucine clearly indicates that the leucine regulator gene is present and active in the suppressor mutants. Therefore the *su leu 500* locus must represent something other than the leucine regulator.

A modification of the above hypothesis seems to explain all the observations which have been made thus far. The *leu 500* mutation can be pictured as having altered the specificity of the leucine operator so that the repressor for an entirely different system could act upon it. This "foreign" repressor might even act as a superrepressor on the leucine operator. In any event, the presence or absence of leucine would not affect the activity of the "foreign" repressor. The *su leu 500* locus would then be the regulator gene controlling the "foreign" repressor, and any mutations which removed or inactivated this gene would relieve the O° condition of the leucine operon. Another effect of the suppressor mutations would be to put into a constitutive state the operon(s) which this "foreign" regulator gene normally regulates.

Implicit in the O° designation of Jacob and Monod[1] is the concept of a mutation which affects the transcription-initiating capacity of the operator. The hypothesis proposed here considers a mutation which primarily affects the specificity of the operator's response to repressor. Since evidence presented above indicates that the operator bearing the *leu 500* mutation is still capable of initiating transcription, it might be more accurate to use O^x as the designation for this type of mutation.[18]

The low levels of leucine pathway enzymes in the suppressed mutants could be attributed to the altered state of the leucine operator, owing to the continued presence of the *leu 500* mutation. This latter hypothesis is supported by Burns' observations on the leucine enzyme levels in the *su leu 500* strains in which the altered leucine operator (*leu 500*) was replaced by the wild-type operator. He found that the enzyme levels were about the same as in the wild-type strain.[17]

It is interesting to note that the *leu 500* mutation arose in a culture treated with 5-bromouracil. When tested for the pattern of reversion induction by 2-aminopurine, in a manner described previously,[19] it gave evidence of being the result of a transition of the bases of a single nucleotide pair. Should the hypothesis we have proposed be borne out, it will mean that a quite radical alteration in the specificity of the operator was achieved by this single base-pair change. If this is not a unique case, then the implication would be that some operators differ in specificity, with respect to their repressors, by a relatively small number of nucleotides.

We wish to express our gratitude to Mrs. Bernadine P. Miller, Miss Doris A. Blume, and Mrs. Susan S. Emmons for untiring and able assistance with the experiments.

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*ENZYMES OF DNA SYNTHESIS IN NUCLEI OF SEA URCHIN EMBRYOS**

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Thus far, the only enzyme system known to be capable of synthesizing DNA in the presence of a DNA primer is the system originally discovered by Kornberg and his associates and named DNA polymerase.[1] Since then, enzymes of the same type have been detected in cells of higher organisms.[2, 3] A simple expectation would be that this enzyme is a constituent of cell nuclei, where the synthesis of DNA takes place. This seemed doubtful until quite recently. In mammalian tissues it seemed to be a relatively soluble enzyme, and several published tests of its nuclear localization did not find the enzyme concentrated in the nuclei.[4, 5] However, a preliminary account of the present work did confirm the association of DNA poly-

merase with nuclei of the sea urchin blastomeres,[6] and evidence of the presence of DNA polymerase in nuclei of mammalian cells has been since published.[7]

In the species of urchin we employed for most of this work, *Strongylocentrotus purpuratus*, the first division takes place synchronously about 120 min after fertilization (at 15°) and the next 5–6 divisions occur at intervals of about 100 min. During each cell generation following the first, 55 min are spent in interphase and about 45 min in mitosis. A detailed schedule is given by Mazia, Harris, and Bibring.[8]

The results of Nemer[9] show that the major portion of the DNA synthetic period during the first few divisions occurs in the early part of interphase. Preliminary results obtained in our laboratory confirm this finding, and it is estimated that the DNA synthetic period lasts 10–15 min. The isolated nuclei from early cleavage stages would therefore be expected to contain a large amount of polymerase, enough to have synthesized close to 10% of the nuclear DNA in one minute. The majority of nuclei would have completed synthesis at the time of isolation, but there is no reason to expect that the presence of polymerase is only transitory, and our results indicate it is not.

Methods.—1. *Isolation of nuclei:* The method described by Hinegardner[10] for isolation of nuclei from sea urchin embryos was followed in detail. At the end of this isolation procedure, the nuclei were at a concentration of 2×10^8 nuclei per ml in a medium of 1.2 *M* sucrose and 0.001 *M* $MgCl_2$. In this medium they could be frozen and preserved at −40° for at least a year without serious loss of activity. At −10° most of the activity was lost in a week.

2. *Enzyme assay:* The DNA polymerase was assayed by the method described by Lehman *et al.*,[11] with some modification of details. This assay measures DNA synthesis as the incorporation of a radioactive deoxyribonucleotide (dTP) into material insoluble in perchloric acid. Through the great kindness of Dr. Arthur L. Kornberg, P^{32}-labeled substrates were available for most of the early experiments; at one time or another, each of the four deoxynucleotide triphosphates was used as the radioactive one. In later experiments, deoxyadenosine-5′-triphosphate-8-C^{14} prepared by Schwarz BioResearch was used.

The reaction mixture now employed to obtain maximum activity contains the following amounts of the various required substances: dATP-C^{14}, 0.15 μmoles; dGTP, 0.15 μmoles; dCTP, 0.15 μmoles; dTTP, 0.15 μmoles; tris-maleate buffer pH 7.4, 80 μmoles; $MgCl_2$, 5 μmoles, or $MnCl_2$, 1 μmole; β-mercaptoethanol, 0.6 μmoles; DNA (when used), 50–75 μg; nuclear preparation, 0.1 ml (2×10^7 nuclei); water to final volume of 0.6 ml. Ordinarily the nuclei were added in the sucrose-Mg^{++} mixture in which they had been stored.

The DNA used as added primer was a high polymer preparation isolated from sea urchin sperm with the use of Duponol followed by the Sevag technique for the separation of protein, and was dissolved in 0.01 *M* KCl. In an experiment in which DNA from *Serratia marcescens* was used, it was isolated by the method of Marmur.[12]

The DNA synthetic reaction was carried out at 37° for 40 min, with shaking. It was terminated by chilling and by adding 1 ml of cold 1 *N* perchloric acid. After 5 min, 1 ml of cold water was added, and the precipitate centrifuged at $4{,}000 \times g$ for 10 min. The precipitate was redissolved in NaOH and reprecipitated with perchloric acid as described by Lehman *et al.* The precipitate was then washed once with 3 ml of 75% ethanol, dissolved in 2 *N* NH_4OH, transferred to a planchet, dried, and the radioactivity measured with a thin-window flow counter.

The absolute data on incorporation given in the figures and tables that follow will show some variation. Part of the variation is biologically significant and is dependent on differences in the developmental stage of the nuclei available for individual experiments, and part is the result of minor changes in experimental procedure. The variation of polymerase during development will be considered in a later publication.

Results.—The nuclei from sea urchin embryos in the early stages of development, as well as from unfertilized eggs, definitely contain DNA polymerase; however, we have found none in sea urchin sperm. In the present report, we shall concentrate on

the nuclear polymerase from the 64-cell up to about the 200-cell embryo. During this period of development, the rate of cell multiplication is fairly uniform, and the yield of isolated nuclei is high.

1. *Requirements for DNA polymerase activity:* The general conditions for maximum activity are similar to those found in other systems. As Figure 1 shows, the pH optimum of the enzyme is around 7.4. The inhibition of the reaction in a medium of increased ionic strength, reported for thymus polymerase,[2] is observed

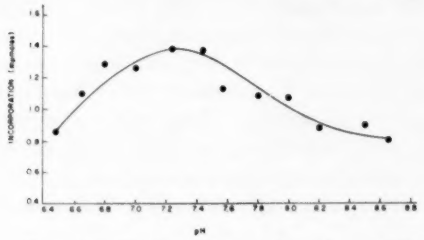

FIG. 1.—pH dependence of nuclear DNA polymerase. Ordinate: mμmoles of C^{14}-deoxyadenylate incorporated by 2×10^7 nuclei. Reaction mixture as given in text, with tris-maleate buffer at pH values shown on abscissa.

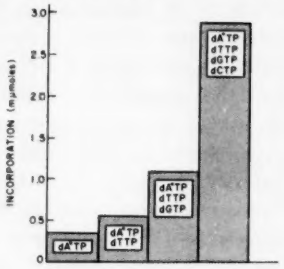

FIG. 2.—Substrate-dependence of incorporation of C^{14}-deoxyadenylate. Assay as described in text.

with the sea urchin enzyme; addition of NaCl to a final concentration of 0.1 *M* reduced the activity in our standard assay by one half. While the protection of the -SH groups of the sea urchin enzyme by a substance such as β-mercaptoethanol does not seem to be an absolute requirement, synthesis is reduced by half in 4×10^{-6} *M* hydroxymercuribenzoate, indicating that -SH groups are required for enzyme activity. Either Mg^{++} or Mn^{++} is required for significant synthetic activity. A Mn^{++} concentration of about one fifth that of Mg^{++} is optimum and gives somewhat better synthesis than Mg^{++}. Calcium does not activate the enzyme at all.

As expected for a DNA polymerase, all four dTPs are required for maximum synthesis (Fig. 2). The finding that some activity is observed when one dTP is supplied, and still more when two or three dTPs are supplied, is not surprising, in view of the fact that we were using whole nuclei which may contain substrates.

2. *Priming:* Because DNA synthesis takes place early in interphase, most of the isolated nuclei would be expected to contain a tetraploid amount of DNA (about 3.6 picogm per nucleus) and might be expected to carry out DNA synthesis without additional primer. This proved to be the case, but nevertheless the addition of high polymer DNA in solution greatly increased the synthesis (Fig. 3).

No sample of high polymer DNA was found to be incapable of priming the reaction over and above the level of "self-priming." This question was tested with sea urchin sperm DNA, DNA isolated from *Serratia marcescens*, salmon sperm, and calf thymus. The sea urchin sperm DNA was tested for its native condition by measurements of its hypochromicity in 0.1 *M* NaCl which yielded a difference in O.D. of 33 per cent between 55° and 75°. Heating to 90° followed by rapid cooling diminished the priming ability of the DNA by about a third. On the other hand, a brief digestion of the DNA with pancreatic DNAase, followed by heating to 70° for several minutes to destroy the enzyme, markedly improved the priming ability (Fig. 4).

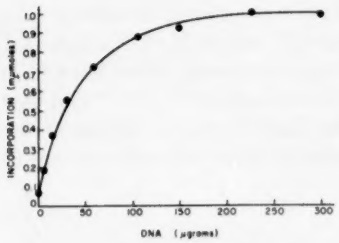

FIG. 3.—Priming of DNA polymerase activity of nuclei by added DNA. The figures for "self-primed" synthesis (no added DNA) are significant. Ordinate: mμmoles of dATP-C^{14} incorporated.

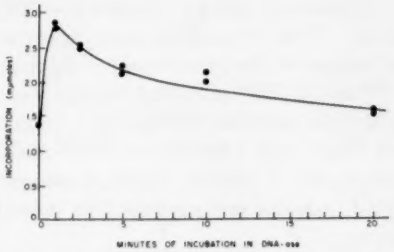

FIG. 4.—Effect of pancreatic DNAase on the priming ability of DNA. The DNA preparation was isolated from sea urchin sperm. A solution (1 mg per ml in 0.01 *M* KCl) was incubated with crystalline pancreatic DNAase (final concentration 1 μgm per ml) at room temperature for the times indicated, then brought to 70° for a few minutes to inactivate the DNAase. In each assay, 0.5 ml of the modified DNA was added to the reaction mixture described in the section on *Methods*.

3. *Magnitude and kinetics:* In the assay system described above, with added DNA as primer, a sample of 2×10^7 nuclei from early division stages will incorporate 3 mμmoles of dAMP-C^{14}, and the best preparations will incorporate up to 5 mμmoles into DNA in 40 min at 37°. This number of nuclei contains about 0.3 mg total protein. The activity thus would correspond to the incorporation of 10 to 17 mμmoles per mg protein. It may be useful to compare a few published data on polymerase activity:

Source	Activity*	Source	Activity*
Sea urchin embryo nuclei (40 min incub.)	10–17	Purified calf thymus enzyme (1 hr incub.)[2]	38
E. coli extract (30 min incub.)[11]	1	Regenerating rat liver nuclei (3 hr incub.)[7]	2
Purified *E. coli* enzyme (30 min incub.)[11]	2000–4000	Extract of regenerating rat liver nuclei ("pH 4.8 precipitate"; 4 hr incub.)[7]	17
Calf thymus extract (1 hr incub.)[2]	0.7		

*Activity here expressed as mμmoles of radioactive nucleotide incorporated into acid-insoluble material at 37° during time indicated.

Inevitably, there is some ambiguity about the meaning of DNA polymerase assays on crude preparations such as our nuclei. It is clear from the kinetic picture of our system, shown in Fig. 5, that degradation of the product can take place. The initial rate of incorporation is not maintained and, after about 45 min, the recovery of incorporated nucleotide actually declines.

The time-course of the synthesis being as complex as it is, there would seem to be no point to a closer consideration of net synthesis at this time. However, the net synthesis represented by the data given above is negligible; it represents up to 10 per cent of the added DNA primer (which primes 90 per cent of the synthesis) and the utilization of almost 3 per cent of the added nucleotides.

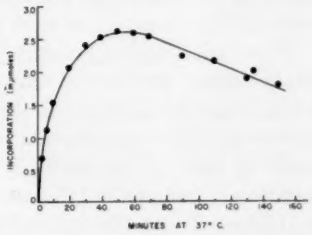

FIG. 5.—Time-course of DNA polymerase reaction. Reaction mixture as described in text.

4. *Synthesis of DNA:* Replicative DNA synthesis has been established by two methods. First, it has been shown that the ratios of nucleotides incorporated correspond to those of the primers used. To this end, we have studied the incorporation of $dATP^{32}$ and $dGTP^{32}$ when the reaction was primed by sea urchin DNA having a GC content of 39 per cent and by DNA from *Serratia marcescens*, which has a GC content of 59 per cent. In these experiments, the incorporation of $dATP^{32}$ and $dGTP^{32}$ was measured in parallel, separate assays. As Table 1 shows, the results are as expected; adenine and guanine were incorporated in ratios very close to that of the primers.

TABLE 1

INCORPORATION OF ADENYLATE AND GUANYLATE BY NUCLEAR POLYMERASE COMPARED WITH PER CENT GC OF PRIMER DNA

Radioactive nucleotide supplied	Primer DNA added	mμmoles nucleotide-P^{32} incorporated	Per cent GC incorporated	Per cent GC in primer DNA†
$dATP^{32}$	none	0.025	36	39
$dGTP^{32}$	none	0.014		
$dATP^{32}$	sea urchin	0.820	37	39
$dGTP^{32}$	sea urchin	0.482		
$dATP^{32}$	Serratia	0.160	58*	59
$dGTP^{32}$	Serratia	0.200		

* Corrected for self-primed synthesis.
† Determined by CsCl gradient centrifugation.[16]
Reaction mixtures: 10^7 nuclei; 0.01 μmole each dATP, dGTP, dCTP, TTP; 2.0 μmoles $MgCl_2$; 0.3 μmole mercaptoethanol; 20 μmoles KH_2PO_4 – K_2HPO_4, pH 7.0; 0.05 mg primer DNA where added; final volume, 0.3 ml.

The second experiment demonstrating replicative synthesis was the ultracentrifugation, in a CsCl gradient, of an incubated reaction mixture containing sea urchin sperm DNA and the newly-synthesized product. Experimental details are given in the legend to Figure 6. It is seen in Figure 6 that the newly-synthesized material has the same average density as the sea urchin DNA present in the nuclei and added as primer, but that it forms a wider band, as though it had a lower average molecular weight.

We conclude that the activity we have been designating as DNA polymerase does in fact catalyze a replicative synthesis of DNA.

5. *Nuclear localization of activity:* All the data presented thus far show the presence of an active DNA polymerase in nuclei. We have been unable to demonstrate the presence of this enzyme in the cytoplasm. At the time of our preliminary report,[6] this seemed to be an ambiguous result, since the cytoplasmic fractions also inhibited the activity of the nuclei, and hence would seem to be untestable for their own activity; but further work has elucidated this inhibitor activity.

If we add varying amounts of an embryo homogenate to nuclei isolated from a similar stage, we observe that small amounts actually increase the yield of DNA, while larger amounts bring about a decreased yield (Fig. 7). The same result is obtained when a supernatant obtained by high speed centrifugation of the homogenate is used. This result is quite intelligible, because it can be mimicked by addition of pancreatic DNAase, and the homogenates would be expected to contain a DNAase.[13] At the optimal concentrations, this DNAase enhances the reaction by partial splitting of the primer, an effect shown in Figure 4. At a sufficiently higher concentration, one would expect destruction of the primer, the product,

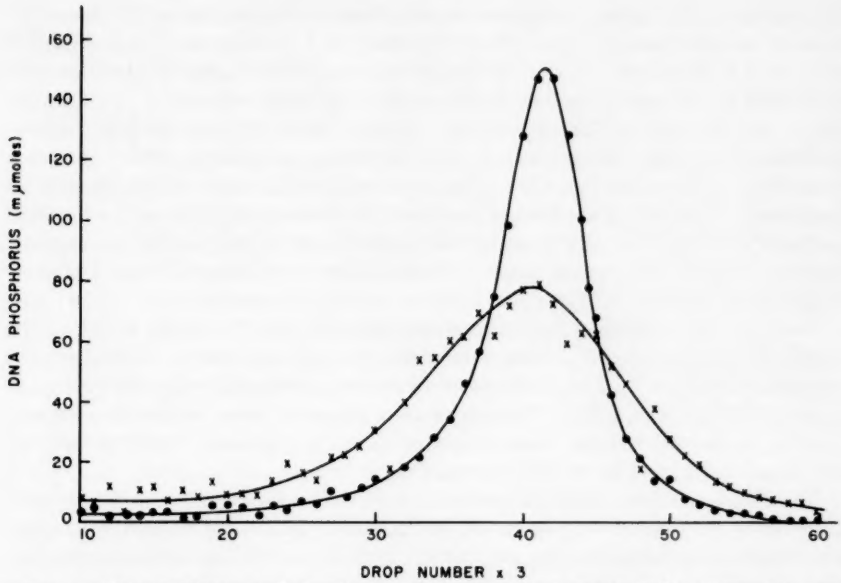

FIG. 6.—CsCl equilibrium centrifugation of the synthesized and primer DNA. Assay tube contained 1 × 10^6 nuclei and the appropriate amounts of the other required constituents. After incubation with $dGTP^{32}$ as the radioactive nucleotide, the mixture was centrifuged at 10,000 rpm for 5 min, .01 gm of activated charcoal was added to remove the free nucleotides, and after 15 min the charcoal was removed by centrifugation. Enough CsCl was added to bring the concentration to 5.7 molar, and this solution was centrifuged in a Spinco Model L ultracentrifuge for 48 hr at 35,000 rpm. Fractions of three drops each were collected after puncturing the bottom of the tube. These were assayed for UV absorption at 260 mμ and radioactivity. ●—DNA phosphorus calculated from the UV absorption. ×—synthesized DNA phosphorus determined by radioactivity and adjusted to give a cure of the same area as the UV curve.

or both; a low yield in the polymerase assay is to be expected, and Figure 7 shows this was found.

Thus far, then, we have no evidence to suggest that the sea urchin embryos contain DNA polymerase outside their nuclei. When we speak of the localization of

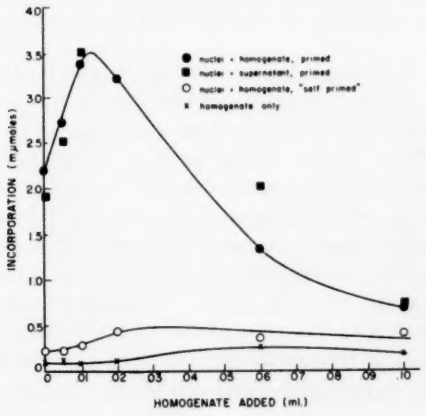

FIG. 7.—Effect of an homogenate of sea urchin embryos (*S. purpuratus*) on the DNA polymerase activity of nuclei. The nuclei were isolated from 165-cell embryos. The homogenate was made from embryos at a slightly earlier stage (*ca.* 100 cells per embryo). The homogenate contained about 15 mg of protein per ml. It is seen that the homogenate itself shows a very small activity, which can be attributed to the number of nuclei present. Ordinate: mμmoles deoxyadenylate-C^{14} incorporated.

the enzyme in the nuclei, we can be referring only to its retention under the conditions of isolation and storage. We have found that the enzyme is not diffusible into the 1.2 *M* sucrose solution in the presence of 0.001 *M* Mg^{++}; this has been confirmed by repeated washing of the nuclei with large volumes of the sucrose-Mg^{++} mixture without loss of activity. If Ca^{++} is substituted for Mg^{++} in the isolation, the enzyme is also retained, but Mg^{++} must be added in order to measure its activity. These are the only conditions found thus far under which the activity is retained. The nuclei are not active if they are isolated in 0.1 *M* KCl, which will partially substitute for Mg^{++} during the isolation, or if they are dialyzed against water or washed with sucrose alone. We do not know whether the loss of activity in the latter instances implies a loss of the enzyme or its inactivation.

To check the possibility that the activity isolated with the nuclei might be the result of the adsorption of a soluble enzyme under the conditions of isolation, the method of Behrens,[14] in which isolation of nuclei is accomplished entirely in non-aqueous media, was tried. The nuclei thus prepared were as active as nuclei isolated in sucrose from the same embryos. The "self-primed" synthetic activity was somewhat higher in the Behrens nuclei.

The attachment site of the polymerase within the nuclei has not been determined. If embryos are treated with estradiol, which Agrell[15] has found to have colchicine-like effects, nuclei can be prepared which have a doubled chromosome number. These nuclei contain twice as much DNA as the controls and have twice the polymerase activity (Table 2). Thus, the polymerase activity of a nucleus is exactly proportional to the number of chromosome sets, but, since nonchromosomal constituents of the nuclei may also increase in polyploidization, the experiment is not a proof of the chromosomal localization of the enzyme.

TABLE 2

Nuclei*	DNA per nucleus (mg)	mμmoles TTP^{32} "self-primed"	Incorporated primer
Control 1 (12 hr; *ca* 110 cells)	2.7×10^{-9}	0.054	0.76
Control 2 (16 hr)	2.8×10^{-9}	0.041	0.65
Estradiol (12th–16th hr.)	5.9×10^{-9}	0.097	1.27

* 1×10^{-7} nuclei per assay.
Evidence for doubling of DNA polymerase with doubling of DNA. Embryos were exposed to estradiol (1 mg per liter of culture) at 12 hr of development (at 15°; *ca* 100-cell stage) and the exposure continued until the 16th hr, at which time nuclei were isolated. Control nuclei were isolated at the 12th and 16th hr of development in normal sea water. The difference between the polymerase activity of the controls at 12 and 16 hr is probably significant; this is the time at which the rate of cell division begins to decline sharply.

6. *Other enzymes of DNA synthesis:* If we accept the fact that DNA is replicated in the nuclei, we can expect that DNA polymerase must operate there. But the substrates for the terminal polymerase reaction could be made anywhere in the cell, as long as they are available to the nucleus. In the present work, we have tested our nuclei for the presence of the enzymes of the penultimate step of DNA synthesis, the deoxynucleotide kinases which form deoxynucleoside triphosphates from monophosphates and ATP. The presence of such enzymes in the isolated nuclei is demonstrated by experiments such as the one reported in Figure 8, where it can be seen that monophosphates alone are incorporated into DNA. In all experiments thus far, the maximum synthesis obtained with the monophosphates and ATP is less than that obtained with triphosphates, as though the kinases could not supply triphosphates as fast as the triphosphates can be incorporated into DNA.

These results are quite in harmony with earlier findings which show that animal nuclei can even incorporate nucleosides into DNA.[16]

Discussion.—In the sea urchin embryos we have studied, all of the DNA polymerase seems to be localized in the nucleus and to be strongly bound by nuclear structures as long as an adequate level of Mg^{++} or Ca^{++} is maintained. By contrast, similar enzymes are readily extractable from mammalian tissues, and are not recovered in the nuclei by ordinary isolation procedures.[2-4] Keir, Smellie, and Siebert[7] have recovered polymerase in nuclei isolated from rat liver under nonaqueous conditions, but these cells also seemed to contain extranuclear polymerase, and the enzyme was readily extractable from the nuclei. The contrasting results on nuclear localization and retention of the enzyme might be attributable to methods of isolation. However, it is interesting to consider that the differences in the localization of the enzyme may reflect its operations in the cell. The sea urchin blastomeres are synthesizing DNA at a high rate, and all the nuclei isolated are either in the act of synthesis or have just completed it. It is at the time of DNA replication that the enzyme would be expected to be associated with DNA, and it is conceivable that the enzyme is present in another form, perhaps a soluble form, during long G_1 or G_2 periods.

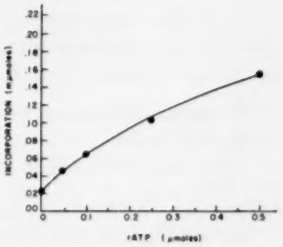

FIG. 8.—Dependence of DNA synthesis on ATP concentration when deoxynucleotide monophosphates are used as substrate. Thymidine monophosphate C^{14} was used as the radioactive nucleotide in the standard assay mixture. The micromoles of ATP added to this mixture are designated on the abscissa.

Even though the sea urchin nuclei contain DNA and polymerase, the level of "self-primed" synthesis is only about 10 per cent of the synthesis obtained when an amount of free DNA equivalent to the amount present in the nucleus is added. The "self-primed" synthesis remained low even when the nuclei were exposed to a DNAase which enhanced the priming activity of added DNA (Fig. 7). If, as it would seem, the decisive difference between the synthesis "self-primed" by chromosomal DNA and synthesis primed by dissolved double-stranded DNA is precisely the fact that the latter is in solution, the variable may involve the mutual access of DNA and polymerase. Presumably, the completion of replication of a given chromosome region makes it inaccessible to further effective association with the enzyme, since each segment of the DNA is thought to replicate only once in each cell generation. Perhaps the limited "self-primed" synthesis reflects the fact that most of the DNA synthesis takes place quite early in the interphase in the sea urchin egg, in which case most of the DNA in the nucleus is in chromosome regions that have already replicated.

Summary.—Nuclei isolated from sea urchin embryos during early cleavage stages, a period of development during which the number of nuclei doubles every two hours or less, contain a high level of DNA polymerase. There is no evidence of the presence of the polymerase outside the nuclei. The properties of the enzyme activity itself are very similar to those observed with DNA polymerase from other sources. The nuclei also contain kinases permitting the incorporation of deoxynucleoside monophosphates into DNA in the presence of ATP. The "self-primed" synthesis, which depends on the priming of the polymerase of the nuclei by the

DNA of the same nuclei, is small compared to the synthesis obtainable when free DNA, native by usual standards, is added as primer. The base ratio of the product is that expected from the base ratio of the primer.

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AN APPROACH TO THE MEASUREMENT OF GENETIC RELATEDNESS AMONG ORGANISMS

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The presence of genes in common may be taken as a guide, not only to taxonomic relationships among organisms, but also to probable evolutionary relationships. However, reproductive isolation of distantly related forms precludes the use of the usual methods of genetics for the determination of gene similarities.

According to contemporary understanding the nucleotide sequences in deoxynucleic acids (DNA) represent the total genetic potential of organisms, and sequences held in common are indicative of similar genes. Since this chemical basis of heredity appears firmly established, the *in vitro* detection of genetic homology by physical chemical means is a clear possibility. Moreover, a distinction can be made between the *presence* and the *activity* of genes when similarities and differences among the primary gene products, messenger RNA molecules,[1] are also compared. Thus, RNA molecules which interact with DNA indicate phenotypic similarities while cross-reacting DNA molecules reveal genotypic similarity.

Similarities between two groups of DNA molecules have, in fact, been demonstrated[2, 3] by duplex strand formation in which the two partners of the new du-

plex originated from different, although genetically related, sources. In these experiments the density gradient equilibrium centrifugation method was used to detect the duplex molecules. So far, however, this method has shown neither the scope nor the convenience required for routine application.[4]

In the paragraphs below, it will be shown that the use of the DNA-agar technique for the study of complementary interactions between DNA and RNA from different organisms allows more extensive measurement of genetic homology. In this procedure, denatured DNA is immobilized in a solid phase by trapping it in a gel of agar.[5] It is then unable to renature but is still available for complementary interactions with other free nucleic acid molecules. After incubating the DNA gel preparation with free RNA or denatured DNA molecules, those molecules which formed a stable duplex with the trapped molecules may be separated from the rest by the simple expedient of washing the gel. The bound molecules may then be recovered by changing the ionic conditions or heating so that the hydrogen bonds between the strands of the duplex are decomposed. The method can be applied to quantitative comparisons of the amount of homologous and heterologous RNA or DNA bound to DNA embedded in agar.

Materials and Methods.—Bacterial cultures were grown in a nutrient broth medium except for *Escherichia coli* and *Aerobacter aerogenes* which were grown in a minimal glucose salts medium. RNA was prepared and purified by methods already described.[5, 6] Pulse-labeled RNA was prepared from cells exposed to C^{14}-uracil for 1 min in the minimal medium.

DNA was prepared by the procedure of Marmur.[7] Labeled DNA was prepared from cells grown for several generations in either C^{14}-uracil or $P^{32}O_4^{-}$. The labeled DNA was sheared by passing it through a French pressure cell at 10,000 p.s.i. at 1 mg/ml in 2 × SSC (SSC is 0.15 *M* NaCl, 0.015 *M* Na citrate. The resulting molecular weight is about 500,000.[8] DNA, either native or sheared, was denatured by heating for 5 min at 100°C in 0.1 × SSC at concentrations of 0.1–1.0; mg/ml.

Denatured DNA was trapped in agar as previously described.[3] Five ml of 0.1 × SSC containing 1–5 mg of denatured DNA was thoroughly mixed at about 90°C with 5 ml of 6% agar-agar number 3 (Oxoid, Ltd.) and rapidly chilled. The resulting gel was twice pressed through a 40 mesh screen and washed at 60°C with 2 × SSC. It was stored at 4°C until required. The entrapped DNA was assayed by its ultraviolet absorption after dissolving an aliquot of DNA-agar in 5 *M* $NaClO_4$. The organisms, sources of DNA, and the DNA content of the various DNA-agar preparations used are given in Table 1.

For duplex formation, about 0.5 gm wet weight of agar gel containing approximately 100 μg of DNA was mixed with the radio-labeled RNA or sheared, denatured DNA in 1.4 volumes of 2 × SSC and incubated at 60°C for 15 hr in a vial.[5] The agar gel was then placed in a heated glass tube and the resulting column washed with ten 10 ml fractions of 2 × SSC at 60°C over a period of 1 hr to remove unadsorbed nucleic acid. Upon raising the temperature to 75°C and lowering the salt concentration to 0.01 × SSC, the hybridized RNA or DNA could be quantitatively recovered with five further 10 ml fractions.

Results.—(1) *Interaction of E. coli messenger RNA with the DNA of other organisms:* The extent of homology between *E. coli* and a variety of other organisms was studied by means of the reaction of *E. coli* pulse-labeled RNA with DNAs from several species. In the homologous reaction some 30 per cent of the rapidly labeled RNA fraction, equivalent to only 1 per cent of the total RNA, is bound.[5, 8] The unbound portion is mostly ribosomal RNA of low specific radioactivity. This RNA is homologous to such a small portion of the DNA[10] that it contributes little to the bound radioactivity unless the DNA-RNA ratio is extremely high. In Figure 1 are shown the actual data for this experiment as well as for three similar ones in which trapped DNAs from other sources were used. With the DNA of

TABLE 1

ORGANISMS AND DNA SAMPLES USED

Organism	Source	% GC of DNA[4, 9]	Concentration of DNA in agar preparation
Enterobacteriaceae:			
E. coli B	Laboratory strain	50	300 μg/g wet weight
E. coli ML 30	"	50	210 "
E. coli K 12 (λ)	"	50	250 "
Aerobacter aerogenes	ATCC 211	56	320 "
Aerobacter aerogenes	ATCC 13048*	56	250 "
Klebsiella pneumoniae	8821/6*	55	150 "
Proteus vulgaris	ATCC 4669	38	240 "
Salmonella typhimurium	ATCC 7823	50	280 "
Serratia marcescens	ATCC 4180	57	120 "
Serratia marcescens	SM 11*	57	80 "
Shigella dysenteriae/s	*	50	150 "
Other bacteria:			
Aeromonas hydrophila	ATCC 9071	54	190
Bacillus subtilis	ATCC 6051	43	350 "
Pseudomonas aeruginosa	ATCC 9027	66	300 "
T4 bacteriophage	Laboratory strain	35	270 "
Calf thymus	Commercial DNA	41	410 "
Mouse liver	B. H. Hoyer[15]	41	400 "
Salmon sperm	Commercial DNA	41	380 "

* Cultures obtained through the generosity of Dr. M. Mandel, University of Massachusetts. Unless otherwise stated, all other bacterial cultures were obtained from the American Type Culture Collection (ATCC).

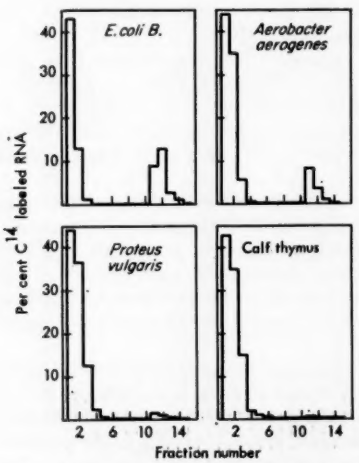

FIG. 1.—Reaction of C^{14}-pulse-labeled RNA from *E. coli* with agar containing DNA from various organisms. 50 μg of RNA prepared from cells labeled for one min with C^{14}-uracil was incubated with 0.5 gm of each DNA-agar, at 60°C for 15 hr. Ten washes were given at 60°C with 2 × SSC. The temperature was then raised to 75°C and five 10 ml fractions of 0.01 × SSC passed through. The percentage of the labeled RNA appearing in each fraction was then determined.

two other organisms generally classified with *E. coli* in the *Enterobacteriaceae*[11] there is appreciable, although lower, binding. An experiment, in which DNA of calf thymus was used, showed no significant reaction (Fig. 1*d*). A list of the results of these and other experiments with various DNA preparations is given in the left-hand columns of Table 2. In the second column the percentage of *E. coli* pulse-labeled RNA bound is recorded as 100, and the heterologous reactions are normalized to the homologous reaction.

A number of features are immediately apparent. The cross reactions are not significant with any of the DNAs of vertebrate origin. This is also true of T4 bacteriophage DNA and two DNAs from different families of bacteria.[11] On the other hand, two other strains of *E. coli* were not distinguishable from *E. coli B*, the source of the labeled RNA. Likewise, the DNA from two strains of *A. aerogenes* gave the same amount of cross reaction with *E. coli* RNA. Within the group of enterobacteria the degree of cross reactions ranged between 90 and 10 per cent.

(2) *Interaction of E. coli DNA with the DNA of other organisms:* Similar reactions are possible where both of the interacting nucleic acids are of the deoxy type, providing a few modifications

TABLE 2

BINDING OF *E. coli* B PULSE-LABELED RNA AND DNA FRAGMENTS TO VARIOUS DNA-AGAR PREPARATIONS

Source of DNA	% labeled RNA bound	% RNA bound relative to *E. coli* DNA	% labeled DNA bound	% DNA bound relative to *E. coli* DNA
E. coli B	27.0	100	39.8	100
E. coli ML 30	28.6	106	...	...
E. coli K 12 (λ)	26.4	98	40.3	101
Aerobacter aerogenes 211	13.1	48	20.4	51
Aerobacter aerogenes 13048	14.3	53	17.9	45
Klebsiella pneumoniae	5.7	21	10.2	25
Proteus vulgaris	3.0	11	5.5	14
Salmonella typhimurium	23.5	87	27.9	71
Serratia marcescens 4180	2.1	8	2.8	7
Serratia marcescens S.M. 11	1.6	6	...	...
Shigella dysenteriae	23.8	88	27.7	71
Aeromonas hydrophila	1.2	4	...	...
Bacillus subtilis	0.4	1	...	...
Pseudomonas aeruginosa	0.5	2	0.4	1
T2 bacteriophage	0.3	1	0.4	1
Calf thymus	0.4	1	0.5	1
Mouse liver	0.4	1	...	...

In the left-hand columns are given the results of experiments in which 50 μg of *E. coli* pulse-labeled RNA was incubated with 0.5 gm of the various DNA-agar preparations. Where *E. coli* sheared, denatured DNA was used (right-hand columns), 15 μg was incubated with a quantity of agar containing 150 μg of trapped DNA (about 0.5 gm).

are made. In order that the free DNA may be able to penetrate the agar gel to react with the trapped DNA, it must be sheared to reduce its molecular weight.[8] This has the added advantage that the fragments of DNA may be made to resemble the messenger RNA molecules in size and be roughly of cistron or gene length. All the available evidence suggests that messenger RNA molecules are representative of only one strand of the DNA,[8, 12, 13] while the DNA fragments contain both complements. This difference introduces a complication, for, in the latter case, two complementary strands of DNA can form a duplex molecule in free solution as an alternative to combining with trapped DNA. From a practical point of view, then, it is important that the ratio of trapped DNA to sheared DNA be kept large,[8] and in experiments in which comparison is made among homologous and heterologous reactions that the ratio be constant.

Aliquots of the same DNA-agar preparations used for the RNA experiments were also used for incubations with *E. coli* C^{14}-labeled DNA. The ratio of trapped DNA to sheared DNA was maintained at ten by adjusting the quantity of DNA-agar. The right-hand columns of Table 2 contain the results. Again, animal DNAs show no significant binding, and the bacterial DNAs show cross reactions ranging from 90 per cent to zero. The extent of homology measured in these DNA experiments is in good agreement with that measured by the binding of RNA.

(3) *Specificity of the cross reaction:* The above experiments show partial cross reaction between nucleic acids of different origin. This results from selective reactions on the part of special fractions of these nucleic acids rather than an attenuated reaction of the total material. Evidently, some *E. coli* messenger RNA molecules can find homologous binding sites in a trapped DNA of different origin, while others cannot. Several such cross reactions are described schematically in Figure 2. The rapidly labeled *E. coli* RNA is depicted as a rectangle in which the area is proportional to quantity. The arrows indicate a selection among the labeled RNA molecules by incubation with the DNA-agar of the bacterial species named.

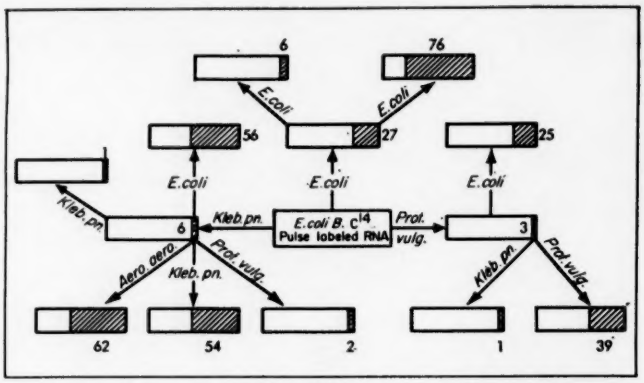

FIG. 2.—Schematic diagram illustrating the selection of different portions of *E. coli* messenger RNA by duplex formation with the DNA of other organisms. The areas of the boxes are proportional to the fraction of *E. coli* C^{14}-pulse-labeled RNA. The fraction which is bound is represented by the shaded area. Arrows represent incubation of the particular fraction from which the arrow originates with DNA-agar of the organism named and point to the resulting distribution into bound and unbound moieties. The upper portion of the diagram depicts reference experiments in which only homologous DNA was used. Thus, in this case the initially bound portion of the labeled RNA is efficiently readsorbed while the unbound portion contains little which is subsequently absorbed under the same conditions.

The shaded area is proportional to the bound portion and the remainder to that which remains in solution.

The upper part of the diagram shows the results of reference experiments in which only *E. coli* DNA-agar was used. It shows that 27 per cent of the labeled RNA is retained by the DNA-agar. This portion when reincubated after DNAase treatment and concentration binds to the extent of 76 per cent, while the initially unbound portion contains only a little hybridizable RNA. Together these experiments show that the messenger RNA fraction represents some one third of the total labeled RNA and that the initial selection is about 80 per cent efficient.[5] The amount of DNA-agar used in each experiment was adjusted to maintain comparable RNA: DNA ratios.

The other two parts of Figure 2 describe experiments in which a smaller portion of *E. coli* messenger RNA is selected on the DNA of either *Klebsiella pneumoniae* or *Proteus vulgaris*. When the bound and unbound material is reincubated with the same DNA, it becomes clear that the RNA which was hybridized is a special fraction of the total. The unadsorbed material now contains little which will bind to the heterologous DNA in question, although it retains its ability to bind to the parent *E. coli* DNA. These selected portions of the messenger RNA will now bind to the heterologous DNA almost as efficiently as to *E. coli* DNA.

Once these special fractions of *E. coli* messenger RNA had been obtained, it was possible to go one step further. The question could then be asked whether the overlap in DNA sequences between *E. coli* and *P. vulgaris* and between *E. coli* and *K. pneumoniae* was the same or different. For this purpose, the *E. coli* RNA selected on *K. pneumoniae* DNA was tested against *P. vulgaris* DNA. Similarly, the RNA selected on *P. vulgaris* DNA was reacted with *K. pneumoniae* DNA. In each case the results were negative, indicating that both organisms have genetic features in common with *E. coli* but that these are nonoverlapping. The RNA selected on *K. pneumoniae* was also tested against *A. aerogenes* DNA with positive

results, indicating that in this case the homology between *K. pneumoniae* and *E. coli* is shared by *A. aerogenes*.

(4) *Agreement of reciprocal cross reactions:* For purposes of quantitative evaluation of genetic relatedness, it is important to know how well reciprocal reactions agree. Thus, the reaction of *E. coli* RNA or DNA with the DNA of *A. aerogenes* indicates a 50 per cent cross reaction. The question remains whether the same conclusion would be reached by study of the inverse reaction. A mixture of two sheared denatured DNAs was made—*E. coli* DNA labeled with C^{14} and *A. aerogenes* with P^{32}. This mixture was then incubated in parallel with the DNA-agar of *E. coli* and *A. aerogenes*. The ratios of the amounts of each DNA bound in the two experiments indicate the extent of the agreement. These experiments are described in Figure 3, and show that in one case the ratio of C^{14}– to P^{32}-labeled DNA is 2, while in the other case it is 0.5. Thus, there is good agreement between the two estimates of the fraction of overlapping sequences in the two DNAs. It is perhaps worth noting that this is not a *necessary* result, since the total complement of DNA may well differ from one organism to another. In each of the two parts of Figure 3, it appears that the heterologous DNA is rather more easily displaced from the DNA-agar than the homologous molecules. Perhaps the fragments of heterologous DNA are not perfectly matched to the immobile DNA strand, and the resulting structure is less stable.

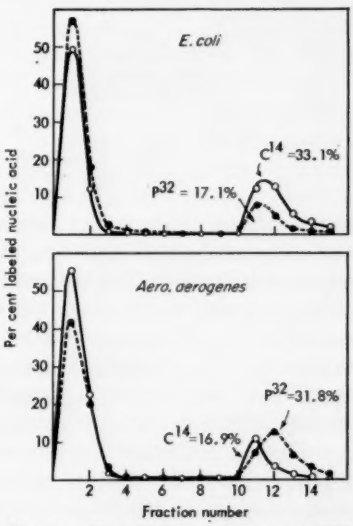

FIG. 3.—Reaction of a mixture of DNA fragments of *E. coli* (C^{14}-labeled) and of *A. aerogenes* (P^{32}-labeled) with the DNA-agar of each organism. 35 μg of C^{14}-labeled DNA and 25 μg of P^{32}-labeled DNA were incubated with 0.5 gm of each of the DNA-agar preparations at 60°C for 15 hr. Fractions were collected as in Fig. 1.

(5) *Relationship of A. aerogenes with other organisms:* In a series of organisms such as those studied here, much more information results from the use of more than one DNA as the reference. This is indicated by experiments testing the cross reaction between *A. aerogenes* nucleic acids and the various trapped DNAs (Table 3). It is apparent that *K. pneumoniae* is more closely related to *A. aerogenes* than to *E. coli*, and that there is little, if any, cross reaction with *P. vulgaris*. In contrast, *P. vulgaris* does cross-react with *E. coli*. On the other hand, the closely interrelated Escherichia-Shigella-Salmonella group are more or less equally related to Aerobacter. The reaction with *A. hydrophila* may suggest that this organism should be classified with the enterobacteria.

(6) *Competition reactions:* If two populations of nucleic acid molecules are related, they should compete for the same binding sites in a DNA molecule. Thus, in the reaction of labeled RNA or DNA fragments with homologous trapped DNA, binding of the labeled nucleic acid should be inhibited by the presence of other unlabeled yet related molecules. Table 4 shows the percentage of *E. coli* P^{32} pulse-labeled RNA which binds

TABLE 3

BINDING OF *A. aerogenes* PULSE-LABELED RNA AND DNA FRAGMENTS TO VARIOUS DNA-AGAR PREPARATIONS

Source of DNA	% labeled RNA bound	% bound relative to *A. aerogenes* RNA	% labeled DNA bound	% relative to *A. aerogenes* DNA
Aerobacter aerogenes 211	31.2	100	25.7	100
Aerobacter aerogenes 13048	30.3	97	27.0	105
Escherichia coli B	16.2	52	12.6	49
Klebsiella pneumoniae	13.2	42	13.8	54
Proteus vulgaris	0.8	2	0.6	2
Salmonella typhimurium	17.4	56	15.5	60
Serratia marcescens 4180	...	...	2.8	11
Shigella dysenteriae	...	...	11.6	45
Aeromonas hydrophila	1.5	5	3.5	13
Pseudomonas aeruginosa	0.3	1	0.5	2
Calf thymus	0.2	1	...	...
Salmon sperm	...	...	0.4	1

Quantities of pulse-labeled RNA, sheared, denatured DNA, and DNA-agar as described in Table 2.

TABLE 4

EFFECT OF HETEROLOGOUS RNA

Unlabeled RNA	Quantity (mg)	% P^{32}-labeled RNA bound
None		25.5
Escherichia coli	2	14.9
Escherichia coli	10	6.7
Aerobacter aerogenes	10	8.2
Klebsiella pneumoniae	10	12.2
Yeast	10	26.9
Mouse liver	2	24.6

20 μg of C^{14}-pulse-labeled RNA from *E. coli* B was incubated with 0.5 gm agar containing 150 μg of DNA in the presence of various unlabeled RNA preparations.

to the parent DNA in the presence of various preparations of unlabeled RNA. The amount of labeled RNA was very low, so that large excesses of unlabeled material could be added. It is evident that the presence of RNA isolated from yeast or mouse liver had no effect on the binding, whereas that from the enterobacteria was effective. Moreover, these heterologous bacterial RNA preparations are almost as effective competitors as the homologous RNA. However, the two enterobacteria appear more distantly related from direct measurements of heterologous duplex formation. It seems therefore that the competition assay may well reveal similarities in nucleotide sequences which were not detected in the direct measurements.

Discussion.—The results presented demonstrate the potential usefulness of procedures which discern genetic homology at the molecular level. Among bacteria especially, where there exists only the faintest paleontological record and the simplest of all ontogenetic processes, the molecular approach seems most promising for understanding evolutionary relationships. Nevertheless, it might be argued that the mere presence of genes in common is an insufficient basis for judging relationships, since many genes might be dormant and contribute little to the structure or function of an organism. However, the DNA-agar procedure permits quantitative assessment of both potentially active and actually active nucleotide sequences in DNAs. Thus, the results with bacterial nucleic acids are equivalent whether RNA : DNA or DNA : DNA interactions are examined. This observation implies that a quantitatively similar fraction, perhaps all, of the genes in each bacteria are expressed during exponential growth.

The experiments described above have examined nucleic acid interactions within only a single family of bacteria. Wilson and Kaplan[14] have suggested, on the basis of comparative studies of enzyme-antibody reactions, that genetic differences between families of bacteria may be greater than that between the major taxonomic divisions of other organisms. Extension of the sensitivity of the DNA-agar procedures may therefore be necessary before the whole spectrum of bacterial types can be cross-compared. It is of interest that the equilibrium centrifugation method for detecting heterologous DNA duplexes has revealed relationships only among rather closely related forms, such as *E. coli* and *Sh. dysenteriae*,[2] or the T-series of bacteriophages.[3] Successful application of this technique requires that long sequences of nucleotides, extending probably over several contiguous genes, be in register in order to form duplexes which are large enough to band during a reasonable time of centrifugation. The fact that smaller fragments of complementary nucleic acids can be examined, and also that one of the two interacting strands can be restrained from homologous renaturation, accounts for the wider application of the DNA-agar method.

The estimates reported above of nucleotide sequences held in common among bacterial species are somewhat arbitrary. This arises from the fact that only those molecules of RNA or DNA which form duplexes stable to 60°C have been considered to be related. It is to be expected that corresponding sites in the DNAs of two related organisms will exhibit a spectrum of similarities and there is evidence (Fig. 3) that some of the heterologous duplexes are more easily dissociated than corresponding homologous ones. Hence, more information could be obtained by testing the stability of the duplexes under a variety of conditions. The somewhat greater similarity revealed by the competition experiments indicates that the molecules which are rejected in a binding test should not be classed as completely dissimilar. With this modified method more distant relationships may be perceived.

Preliminary experiments[15] with nucleic acids of animal origin have shown that essentially identical techniques can be applied to the study of evolutionary relationships among higher organisms, including mammals. It would appear therefore that the approach illustrated in this report is available for the study of the genetic material of living creatures, no matter how complex they may be.

Summary.—By study of the binding of messenger RNA or DNA fragments to homologous and heterologous DNA immobilized in a gel structure, it is possible to measure quantitatively the genetic relatedness of several strains of enterobacteria. Essentially similar results are obtained from the RNA and DNA experiments. Reciprocal reactions in which the DNAs of the two organisms are interchanged give the same estimate of similarity. In the reaction of *E. coli* messenger RNA with the parent DNA, related RNA molecules originating from other organisms compete for the same sites in the DNA. Applications to the study of evolutionary relationships among bacteria and other organisms are considered.

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*THE ENZYMATIC METHYLATION OF RNA AND DNA, II. ON THE SPECIES SPECIFICITY OF THE METHYLATION ENZYMES**

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There is evidence that methylated bases in DNA and sRNA are not randomly distributed in polynucleotide chains. In wheat germ DNA, the two 6-aminopyrimidines, cytosine and 5-methylcytosine, do not appear to substitute randomly for each other, as determined by chemical analysis of oligonucleotides.[2] This specificity appears to be related to the direct methylation of deoxycytidylate of DNA at the polynucleotide level,[1] as has also been found to apply to the origin of the base, 6-methylaminopurine. These observations are in keeping with the incorporation studies of Bessman *et al.*[3] with the DNA polymerase system. This enzyme readily catalyzes the incorporation of dCMP and 5-methyl dCMP into DNA without distinguishing between these deoxynucleotides, and therefore does not appear responsible for localization of methylated bases. In RNA, the methylated bases are uniquely localized to soluble RNA as indicated by a large body of information.[4, 5] Here, RNA polymerase, the enzyme which appears to synthesize all RNA species from a DNA template of normal cells, lacks specificity in differentiating between methylated bases and normal bases. Thus, for example, ribothymidylate is readily incorporated into RNA in place of uridylate with the same nearest neighbor frequency.[6] However, the distribution of methylated bases of sRNA is not random, and analyses of purified sRNA molecules, specific for particular amino acids, indicate that they contain varied amounts as well as different methylated bases.[7, 8] As in the case of DNA, this specific distribution of methylated base has been explained by the observation that methylation occurs at the polynucleotide level rather than the mononucleotide stage.[9, 1] While with DNA it appears that methylation is catalyzed by a single enzyme, in the case of sRNA many enzymes are involved. To date, 5 different enzymes catalyzing specific methylation reactions with sRNA have been isolated.[1]

The present communication is concerned with the species specificity of enzymes which lead to DNA and RNA methylation. With the enzyme systems which have been studied,[10] methylation of nucleic acids does not occur, using enzymes obtained

from the homologous species. Thus, both systems appear to have a marked species specificity. A similar conclusion regarding sRNA methylation has recently been published by Srinivasan and Borek.[11]

Materials and Methods.—In experiments presented below, crude cell extracts of organisms were prepared by alumina grinding, except for *Micrococcus lysodeikticus* which was disrupted with lysozyme, followed by centrifugation at 100,000 $\times$ *g* for 120 min. The sRNA of each organism was prepared by extraction of the high-speed supernatant solution with phenol,[12] followed by alcohol precipitation and dialysis. All DNA preparations were isolated as described by Marmur.[13] Crude cell extracts used for studies of sRNA methylation were prepared in the presence of 5 μg per ml of crystalline pancreatic DNAase. In addition, all sRNA preparations were first preincubated for 30 min at 37° with DNAase at a similar concentration. In the studies concerned with methylation of DNA, pancreatic RNAase, heated to 80° for 15 min, was included in crude extracts at 5 μg per ml. All DNA preparations were treated with 50 μg of RNAase per ml for 1 hr at 37° during isolation. The preparation of the five purified RNA methylating enzymes and the purified DNA methylating enzyme from *E. coli* will be described elsewhere. All comparisons were performed with equal concentrations of both nucleic acid and enzyme protein, respectively, as summarized in the tables. C^{14}-methyl-labeled S-adenosylmethionine (2.2×10^7 cpm per μmole) was obtained from Tracerlab, Waltham, Mass., and used without further purification or dilution.

The bacteria used in these studies as well as their source were the following: *E. coli* W, *E. coli* K12 W6-58-161, *Staphylococcus aureus*, *Mycobacterium phlei*, *Salmonella typhimurium*, and *Corynebacterium diphtheriae* PW-8 (toxigenic with phage Pd), obtained from this Department. *Micrococcus lysodeikticus* was obtained, as the dry powder, from Miles Chemical Co., Clifton, N. J., *Clostridium pasteurianum* was a generous gift from Dr. J. E. Carnahan of the E. I. du Pont de Nemours and Co., Wilmington, Delaware, while *Lactobacillus delbruckii* was obtained from the American Type Culture Collection.

Results.—Table 1 summarizes results obtained when crude cell extracts prepared from a variety of microorganisms were used as sources of methylating enzymes. All crude cell extracts catalyze the methylation of sRNA derived from the starved *E. coli* auxotroph (relaxed strain). However, when sRNA preparations isolated from normal bacteria are used as methyl group acceptors, there is a specificity in methylation activity. For example, crude cell extract of *E. coli* catalyze tl e methylation of all sRNA preparations with the exception of normal *E. coli* sRNA. With crude cell extract prepared from *M. lysodeikticus* a similar specificity is observed; i.e., there is methylation of all sRNA preparations with the exception of normal *M. lysodeikticus* sRNA. This inability to methylate homologous sRNA is also true of extracts of *L. delbruckii*, *S. aureus*, *Mb. phlei*, etc. In general, the methylation enzymes of a given organism will not catalyze the methylation of homologous RNA. However, it is not the case that the enzymes of one organism will methylate sRNA of all others. For example, crude cell extracts of *C. diphtheriae* catalyze the methylation of both normal and starved *E. coli* sRNA and sRNA from *Cl. pasteurianum*, but are virtually inactive with the other species of sRNA preparations, although crude extracts from all the organisms tested lead to extensive methylation of sRNA isolated from *C. diphtheriae*.

TABLE 1

SPECIES SPECIFICITY OF THE METHYLATION OF sRNA

Source of cell extract	Source of sRNA: Starved *E. coli*	Normal *E. coli*	*M. lysodeikticus*	*Cl. pasteurianum*	*L. delbruckii*	*S. aureus*	*Mb. phlei*	*C. diphtheriae*
	C^{14}-CH_3 group incorporated in 30 min (mμmoles)							
E. coli	0.418	<0.002	0.056	0.093	0.040	0.034	0.087	0.096
M. lysodeikticus	0.087	0.048	<0.002	0.039	0.021	0.017	0.010	0.025
Cl. pasteurianum	0.012	0.003	<0.002	<0.002	0.002	<0.002	0.002	(not done)
L. delbruckii	0.107	0.157	0.008	0.080	<0.002	0.002	0.038	0.072
S. aureus	0.059	0.047	0.010	0.054	0.002	<0.002	0.006	0.031
Mb. phlei	0.130	0.031	0.008	0.050	0.015	0.011	<0.002	0.016
C. diphtheriae	0.090	0.052	<0.002	0.060	<0.002	0.003	0.002	<0.002

The conditions for methylation of sRNA were the same in all cases. The reaction mixtures (0.25 ml) contained: 10 mμmoles of C^{14}-CH_3-S-adenosyl-methionine (2.2×10^5 cpm per μmole), 1 μmole of $MgCl_2$, 2 μmoles of mercaptoethanol, 10 μmoles of triethanolamine buffer, pH 8.0, approximately 500 mμmoles of nucleotides as sRNA from each source, and approximately 115 μg of protein from each crude extract. After incubation, all reactions were terminated by the addition of 0.1 ml of 0.1 *M* sodium pyrophosphate, followed by 0.2 ml of 7% $HClO_4$; the acid-insoluble material was washed twice more with 1% $HClO_4$ containing 0.002 *M* sodium pyrophosphate. The final precipitates were dissolved in 0.2 *M* NH_4OH, decanted into metal planchets, dried, and the radioactivity content measured. Incubation mixtures which served as controls yielded 15 cpm above background.

In Table 2, the ability of the purified sRNA methylating enzymes of *E. coli* to methylate sRNA from different sources is summarized. These enzyme fractions, which are not contaminated with one another, lead to the formation of the following methylated bases: 1-methylguanine (two different enzymes produce this compound and are referred to as guanine I and guanine II enzyme fractions); thymine (catalyzed by the enzyme referred to as uracil enzyme fraction); methylated cytosine derivatives (referred to as cytosine enzyme fraction); and 6-methylaminopurine, 6-dimethylaminopurine, and 2-methyladenine (referred to as adenine enzyme fraction). The results indicate that even the different enzymes of one specific organism, *E. coli*, exhibit specificity in their activities toward different sRNA preparations. Thus, the guanine enzymes are virtually inactive with all the species, with the exception of the action of guanine II enzyme fraction on sRNA from *Cl. pasteurianum* and *C. diphtheriae*. The uracil enzyme fraction catalyzes extensive methylation of sRNA from *Mb. phlei*, *M. lysodeikticus*, and *Cl. pasteurianum;* the cytosine enzyme fraction methylates sRNA from *Cl. pasteurianum* most effectively, while the adenine enzyme methylates sRNA from *C. diphtheriae* to the great-

TABLE 2

METHYLATION OF sRNA SPECIES WITH PURIFIED ENZYMES OF *E. coli*

Base in sRNA methylated by enzyme fraction	Source of sRNA: Starved *E. coli*	Normal *E. coli*	*M. lysodeikticus*	*Cl. pasteurianum*	*L. delbruckii*	*S. aureus*	*Mb. phlei*	*C. diphtheriae*
	C^{14}-CH_3 group incorporated in 60 min (mμmoles)							
(1) Guanine I	0.364	<0.002	<0.002	<0.002	0.005	0.007	<0.002	0.007
(2) Guanine II	0.314	<0.002	<0.002	0.030	0.007	0.004	0.003	0.015
(3) Uracil	0.254	<0.002	0.063	0.220	0.006	0.007	0.130	0.059
(4) Cytosine	0.236	<0.002	0.016	0.206	0.033	0.012	0.020	0.055
(5) Adenine	0.124	<0.002	0.014	0.014	0.010	0.004	0.018	0.080

Reaction mixtures were as described in Table 1 except for the following changes: 10 μmoles of triethanolamine buffer, pH 8.0, was used with enzymes, guanine I, guanine II, and cytosine; 10 μ moles of triethanolamine buffer, pH 8.8, was used with the other two enzyme fractions. The amount of sRNA in each case varied between 635 and 748 mμmoles of nucleotides. The amount of enzyme protein added in each case (in μg) was the following: guanine I, 2.3, guanine II, 1.74, uracil 0.34, cytosine 1.04, and adenine 3.7. After 60 min at 38°, the reaction was terminated and the amount of incorporation measured as described in Table 1.

est extent. It should be noted that the specificity of the enzyme for the base methylated remains the same when different sRNA preparations are used. Thus, for example, the uracil enzyme fraction leads only to thymine production whether starved *E. coli* sRNA or sRNA from *M. lysodeikticus* is used as acceptor, and the adenine enzyme yields the same products whether *E. coli* sRNA or *C. diphtheriae* sRNA is the methyl group acceptor.

In Table 3, results are summarized which indicate a similar type of species specificity with the DNA methylation system. The generalization that homologous systems do not result in detectable methylation appears to be true with DNA as with the methylation of sRNA. Also summarized in Table 3 is the ability of the purified DNA-methylation system of *E. coli* to catalyze the transfer of C^{14} from S-adenosyl-methionine to different species of DNA. It is clear that the specificity observed with crude cell extracts of *E. coli* is also observed with the purified enzyme fraction. This partially purified enzyme fraction leads to the production of both 5-methylcytosine and 6-methyl-aminopurine. The ratio of methylation of these two bases of DNA is unaltered during the purification of the DNA methylating activity (approximately 400-fold).

Discussion.—The results presented here indicate that the enzymatic reaction, in which the methyl group of S-adenosyl-methionine is transferred to either sRNA or DNA is species specific. Thus, in the systems studied here, methylation of sRNA or DNA does not occur, using enzyme preparations from homologous species. The species specificity in the methylation of sRNA is analogous to the difference in specificity reported for attachment of amino acids

TABLE 3

METHYLATION OF DNA

Enzyme source	Starved, *E. coli*	Normal *E. coli*	*M. lysodeikticus*	*C. diphtheriae*	*L. delbruckii*	*Cl. pasteurianum*	*Mb. phlei*	*S. typhimurium*	*S. aureus*
	C^{14}-CH_3 group incorporated in 30 min (mμmoles)								
(a) cell extract from:									
E. coli	0.022	<0.001	0.024	0.026	<0.001	0.006	0.042	0.008	0.016
M. lysodeikticus	<0.001	<0.001	<0.001	0.001	0.001	0.026	0.004	0.001	0.002
C. diphtheriae	0.006	0.015	0.013	<0.001	0.006	0.031	0.015	0.003	0.008
L. delbruckii	0.017	0.004	0.015	0.017	<0.001	0.007	0.034	0.009	0.013
Cl. pasteurianum	0.013	<0.001	0.056	0.041	0.001	<0.001	0.027	0.032	0.049
(b) purified *E. coli* enzyme	0.200	<0.001	0.503	0.428	0.013	0.032	0.705	0.149	0.212

The reaction mixture (0.25 ml) contained: 10 μmoles of Tris buffer, pH 8.0, 1 μmole of $MgCl_2$, 2 μmoles of mercaptoethanol, 10 mμmoles of C^{14}-CH_3-labeled S-adenosyl-methionine (2.2×10^7 cpm per μmole), 150 mμmoles of deoxynucleotides as DNA in each case, and 60 μg of protein from crude extracts. In the experiments with purified enzyme, 5 μg of protein was added, the DNA concentration was reduced to 75 mμmoles of deoxynucleotide, an 10.2 μg of heated RNAase was included in each reaction mixture. All other conditions are as described in Table 1. Cell extracts prepared from *Mb. phlei*, *S. typhimurium*, and *S. aureus* did not lead to detectable methylation of any DNA preparation, and for this reason have not been included in the above table.

to specific acceptor sRNA molecules. A number of workers have shown varying degrees of species specificity of aminoacyl RNA synthesis.[14-16] The species specificity of the methylation enzymes reported here is in accord with the observation that different sRNA molecules, specific for different amino acids, have markedly different concentrations of methylated bases.[7, 8] Such a situation suggests that the methylated bases may be another parameter for distinguishing sRNA molecules.

A similar species specificity is observed with the enzyme system which catalyzes the methylation of DNA. We have recently investigated the intraspecies specificity of the DNA methylation reaction and have found marked difference between strains of *E. coli* DNA and the purified DNA methylating enzyme. Thus, DNA isolated from *E. coli* B and *E. coli* K12 are readily distinguished from one another, i.e., the enzyme isolated from *E. coli* K12 can lead to methylation of DNA isolated from *E. coli* B, but the enzyme from *E. coli* B does not catalyze demonstrable methylation of either DNA. In addition, DNA isolated from phage λ (virulent) is readily methylated by any of the enzymes isolated from various *E. coli* strains including *E. coli* K12 (λ). The possible role of the methylation reaction in host-induced modification of bacteriophage, as well as the immune phenomenon of lysogenic bacteria,[17] are now being investigated. These results, as well as those discussed above, will be published later.

Summary.—The enzymes which catalyze the methylation of DNA and RNA are species specific. Thus, reaction mixtures which contain enzyme and nucleic acid from the same source (homologous systems) result in no detectable methylation, while with heterologous systems, in many instances, methylation of nucleic acid occurs. The different RNA methylating enzymes of a specific organism, *Escherichia coli*, exhibit specificity in their activities toward different sRNA preparations.

Note added in proof: Dr. H. Boman (personal communication) has also demonstrated species specificity of the RNA methylation system.

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[10] We have already encountered one exception to this generalization. The DNA methylating enzyme isolated from *E. coli* strain 15 T^- leads to significant methylation of DNA isolated from all strains of *E. coli*, including its own. These studies of the strain specificity of the methylation reaction will be published at a later time.

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*STABILITY OF NITROGEN-FIXING ENZYMES AND THE REACTIVATION OF A COLD LABILE ENZYME**

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The discovery of methods for preparing active N_2-fixing extracts from *Clostridium pasteurianum*[1] and extension of the methods to other organisms[2–5] has given promise that a highly purified N_2-fixing enzyme system soon may be obtained. The enzyme complex has been separated into N_2 and H_2 activating fractions,[6] but as each is rather labile, we have sought methods to stabilize them.

Materials and Methods.—Cultures of *C. pasteurianum* were grown at 30° in a nitrogen deficient medium[2] (no yeast extract was added and Fe was supplied as the citrate rather than the sulfate). The cells were recovered with a Sharples centrifuge, resuspended in water, and recentrifuged. Cells washed with water at room temperature, compared with cells washed with ice water, gave extracts with almost double the activity per milligram of protein.

Cells were dried and extracted as described by Carnahan *et al.*[1] Two grams of dried cells were mixed with 25 ml of 0.05 *M* phosphate buffer, pH 7.0, and the suspension was shaken in a flask under H_2 for an hr at 32.5°. The supernatant material recovered after centrifugation at 20,000 × *g* for 15 min constituted the N_2-fixing preparation.

N_2-fixing activity was determined by exposing the extracts to 64 atom per cent N^{15} excess N_2 at a pN_2 of 0.05–0.1 atm plus argon to 1.0 atm. Each assay vessel contained 1 ml of the extract with 20–25 mg protein per milliliter, and 1 ml of 0.2 *M*, pH 6.7 cacodylic acid buffer. A solution containing 25–40 mg of sodium pyruvate was placed in the sidearm of the vessel (the optimal concentration of pyruvate was determined for each batch of cells). Alkaline pyrogallol present in the center well brought the total volume to 2.5 ml. The reaction was stopped by transferring the contents of the vessel to a Kjeldahl flask containing 3 ml of concentrated H_2SO_4. The atom per cent N^{15} excess was determined,[7] and in some experiments the activity was established by the method of Mortenson.[8]

Results and Discussion.—Loss of activity: The extracts lose activity even when stored under strictly anaerobic conditions (Fig. 1). When stored under H_2 at −20°, the extracts retain activity longer than at 0°, but purification steps cannot be effected at the lower temperature. As an aid to purification attempts, the influence of various factors on the stability of crude extracts was examined.

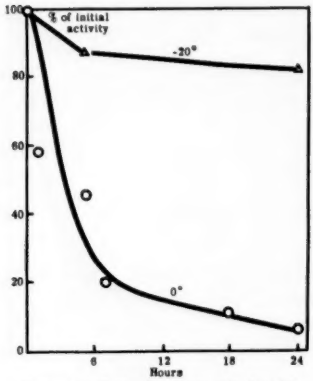

FIG. 1.—Inactivation of the N_2-fixing enzyme complex in extracts of *C. pasteurianum* stored under H_2 at 0° and −20°. Data are plotted relative to the N_2 fixation observed immediately after preparation of the extract.

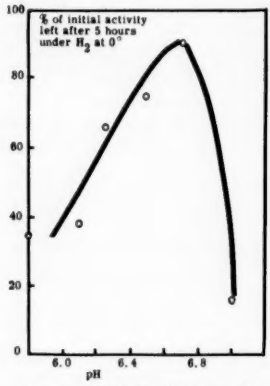

FIG. 2.—Effect of pH on the stability of the N_2-fixing enzyme complex extracted from *C. pasteurianum* and stored under H_2 at 0° for 5 hr.

Buffers, pH, and ionic strength: Aliquots of extracts in 0.05 *M* phosphate buffer were adjusted to different pH's with dilute H_3PO_4 or KOH under anaerobic conditions, and were stored under H_2 in ice. Nitrogen-fixing activity was determined immediately and after 5 hr. Figure 2 shows that extracts were most stable near pH 6.7.

Dried cells were extracted for an hour at 30° under H_2 with water, with 0.06 *M* cacodylic acid adjusted to pH 6.8, or with 0.05 *M*, pH 6.8 phosphate. After centrifugation, the supernatant was adjusted to pH 6.5, flushed with H_2, sealed, and kept in ice. The extract in phosphate buffer retained about 5 per cent of its activity after 20 hr, whereas extracts made with water or cacodylate buffer were completely inactive after 20 hr.

The effect of the concentration of phosphate on stability is shown in Table 1. The extracts had higher initial activity and retained higher activity at the lower phosphate concentrations. The optimal concentration tested was 0.01 *M;* because the capacity of such a buffer is very limited, extracts for most experiments have been made in 0.05 *M* phosphate.

Protein concentration: Extracts with a high concentration of protein were more stable than dilute extracts. When 1 gm of cells was extracted with 10 ml of phos-

TABLE 1

EFFECT OF PHOSPHATE CONCENTRATION ON THE STABILITY OF THE N_2-FIXING ENZYME COMPLEX

Concentration of phosphate	pH	Atom % N^{15} excess	
		Initial	After 5 hr
0.2 *M*	6.5	0.000	0.000
0.1 *M*	6.5	0.540	0.510
0.05 *M*	6.5	0.792	0.784
0.01 *M*	6.5	1.250	1.201
0.001 *M*	6.4	0.768	1.004
No buffer added	6.2	0.887	0.318

Extracts from *C. pasteurianum* were tested immediately and after storage at 0° under H_2 for 5 hr. Fixation of N_2^{15} was for 1 hr.

phate buffer, the supernatant had 20–27 mg of protein per milliliter. These conditions for extraction seemed optimal, because more cells than this yielded smaller volumes of extract without a corresponding increase in protein concentration. Added gelatin, egg albumin, or bovine serum albumin inhibited the initial N_2-fixing activity of the extracts; gelatin and egg albumin had a very minor stabilizing effect which was insufficient to counter their inhibitory action.

Inorganic ions and chelating agents: Added Mg and Ca ions did not improve the stability of the extracts. Extracts were stored with ethylenediaminetetraacetic acid (EDTA) added to chelate heavy metals. EDTA at concentrations of 0.01, 0.001, and 0.0001 *M* inhibited initial N_2 fixation by 82, 45, and 11 per cent, respectively, and did not improve stability.

Glycerol and sucrose: Glycerol[9] and sucrose increase the stability of certain proteins. However, in concentrations from 0.1 to 0.0001 *M* they did not stabilize the activity for N_2 fixation in extracts from *C. pasteurianum*.

Adsorbents and heating: Because of the possibility that inactivation of the enzymes was the result of proteolysis, attempts were made to remove proteolytic enzymes by adsorption or to denature them with heat. Aliquots of extracts were shaken with calcium phosphate gel, bentonite, or kaolin for 30 min at 0,° centrifuged, and the supernatants were kept in an ice bath under H_2. Other aliquots of extract were heated to 50 or 55° for 10 min and then were centrifuged; the supernatants were removed and stored under H_2 in an ice bath. Neither heating nor adsorption improved the stability of the preparations (Table 2), although heating or treatment with calcium phosphate gel improved the initial activity somewhat.

Reducing agents: The effect of sulfhydryl and other reducing agents on the stability of the extracts stored under H_2 at 0° was investigated, and the results are recorded in Table 3. None of these agents improved stability markedly, although ascorbic acid, $Na_2S_2O_4$, mercaptoethanol, 2,3-dihydroxybutane-1,4-dithiol, and glutathione conferred some stability at the levels recorded. Concentrations higher than those recorded were generally inhibitory. Ascorbic acid appeared to be the most useful as a stabilizing agent, but its protection was inconsistent.

The extracts retained their nitrogen-fixing activity only when stored under strictly anaerobic conditions. Figure 3 indicates the rapid loss of activity when a stagnant extract was exposed to air in a test tube. Virtually all activity was lost within 2 hr at 20.° Because of the difficulties posed by complete exclusion of

TABLE 2

EFFECT OF HEATING AND ADSORBENTS ON STABILITY OF THE N_2-FIXING ENZYME COMPLEX

Experiment	Treatment	Atom % N^{15} Excess Initial	Atom % N^{15} Excess After 24 hr	Experiment	Treatment	Atom % N^{15} Excess Initial	Atom % N^{15} Excess After 24 hr
1	Control	0.617	0.041	2	Control	0.453	0.012
	Heated at 50° for 10 min	0.719	0.038		Bentonite, 10 mg/100 mg protein	0.497	0.009
	Heated at 55° for 10 min	0.673	0.004		Bentonite, 20 mg/100 mg protein	0.560	0.013
	Ca phosphate gel, 22.2 mg/100 mg protein	0.756	0.024		Kaolin, 10 mg/100 mg protein	0.475	0.008
	Ca phosphate gel, 33.3 mg/100 mg protein	0.879	0.031		Kaolin, 20 mg/100 mg protein	0.485	0.005

Extracts from *C. pasteurianum* were tested immediately after treatment and after storage at 0° under H_2 for 24 hr. Fixation of N_2^{15} was for 1 hr.

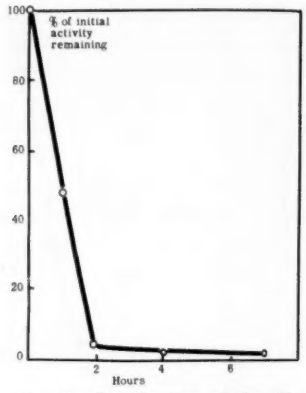

FIG. 3.—Inactivation of the N_2-fixing enzyme complex by oxygen. An extract from *C. pasteurianum* was stored in a test tube in air at 20° without agitation.

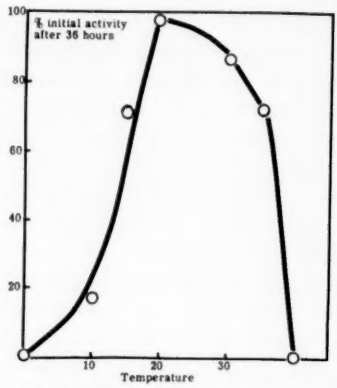

FIG. 4.—Effect of temperature on the stability of the N_2-fixing enzyme complex from *C. pasteurianum* stored under H_2 for 36 hr.

O_2 during purification, compounds were sought which would protect the extracts against inactivation by O_2. Table 4 shows that $Na_2S_2O_4$ and reduced lipoic acid were helpful (about 50 per cent of the activity was retained for 5 hr in air), but that the other compounds tested were ineffective in preventing inactivation.

Enzymes often are more stable in the presence than in the absence of substrate. Extracts from *C. pasteurianum* fix N_2 most effectively in the presence of pyruvate which serves as an energy source and hydrogen donor. Pyruvate did not protect against inactivation by O_2, although the extracts evolved H_2 vigorously from pyruvate and thus aided in displacing O_2.

Optimum temperature for stability: The fact that cells lose activity if they are frozen during drying[1] suggested that an enzyme of the N_2-fixing complex might

TABLE 3

EFFECT OF REDUCING AGENTS ON THE STABILITY OF THE N_2-FIXING ENZYME COMPLEX

Experiment	Treatment	Atom % N^{15} excess Initial	Atom % N^{15} excess After 20 hr
1	Control	0.655	0.022
	Ascorbic acid, 0.001 *M*	0.637	0.115
2	Control	1.056	0.023
	Ascorbic acid, 0.004 *M* + 0.001 *M* EDTA	1.373	0.000
3	Control	0.297	0.035
	$Na_2S_2O_4$,* 0.001 *M*	0.289	0.074
4	Control	0.965	0.036
	$Na_2S_2O_3$, 0.0001 *M*	0.474	0.000
5	Control	0.684	0.127
	Mercaptoethanol, 0.0001 *M*	0.602	0.138
6	Control + 0.00001 *M* vitamin B_{12}	0.896	0.096
	Mercaptoethanol, 0.001 *M* + vitamin B_{12}, 0.00001 *M*	0.894	0.098
7	Control	0.778	0.015
	British antilewisite, 0.001 *M*	0.468	0.000
8	Control	1.034	0.048
	2,3-dihydroxylbutane-1,4-dithiol, 0.00063 *M*	0.951	0.128
	Sodium thioglycolate, 0.001 *M*	0.294	0.011
	Glutathione, 0.001 *M*	1.054	0.083
	Cysteine, 0.001 *M*	0.965	0.036

* Adjusted to pH 7 under H_2 just before use.

Extracts from *C. pasteurianum* were tested immediately and after storage at 0° under H_2 for 20 hr. In each experiment the most favorable result is recorded; although not recorded, concentrations of reagents normally were varied from 0.1 to 0.0001 *M*.

TABLE 4

PROTECTION AGAINST INACTIVATION OF THE N_2-FIXING COMPLEX BY OXYGEN

Experiment	Treatment	Atom % N^{15} excess Initial	After 5 hr	Experiment	Treatment	Atom % N^{15} excess Initial	After 5 hr
1	Control	0.674	0.036	2	Control	0.791	0.014
	Mercaptoethanol, 0.0033 *M*	0.621	0.005		$Na_2S_2O_4$, 0.004 *M*	0.885	0.419
	Mercaptoethanolamine, 0.003 *M*	0.804	0.004		2,3-dihydroxybutane-1, 4-dithiol, 0.004 *M*	0.442	0.086
	Cysteine, 0.003 *M*	0.606	0.018		Reduced lipoic acid, 0.0003 *M*	0.713	0.377
	Sodium thioglycolate, 0.01 *M*	0.828	0.011				

Extracts from *C. pasteurianum* were tested immediately and after storage for 5 hr of approximately 10 ml of extract in an 18 mm diameter test tube at 20° in air without agitation.

be cold labile. Cold lability has been reported by Pullman *et al.*[10] for ATPase from beef heart mitochondria and by Shuster and Doudoroff[11] for D(−)β-hydroxybutyric acid dehydrogenase from *R. rubrum*.

An extract from *C. pasteurianum* initially fixed 0.736 atom per cent N^{15} excess. The extract then was flushed with H_2 and sealed; after storage at 30° for 12 hr it fixed 0.718, and after 36 hr 0.666 atom per cent N^{15} excess. The extracts were recentrifuged after storage and before testing. About 90 per cent of the original N_2-fixing capacity was retained for 36 hr at 30° in contrast to the rapid loss of activity at 0° shown in Figure 1. Activity then was tested after storage of extracts for 36 hr under H_2 over a range of temperatures. Figure 4 indicates that the extracts retained their activity best when stored near 20°.

Reactivation of enzymes: Inactivation effected by O_2 could not be reversed. However, preparations which had lost their ability to fix N_2 after storage at 0° under H_2 for 24 hr could be reactivated by incubation under H_2 at a higher temperature. Figure 5 indicates the percentage recovery at various temperatures; the optimal temperature for reactivation was 22°. Figure 6 shows the percentage recovery with time when the inactivated extract was incubated at 22° under H_2. Reactivation was incomplete but continued to increase between the 3 and 7 hr samplings. However, when extracts were stored in ice for 6 rather than 24 hr, they could be completely reactivated by incubation for 1 hr under H_2.

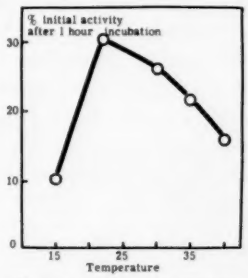

FIG. 5.—Effect of temperature on reactivation of the N_2-fixing enzyme complex which had been reduced to 2% of its initial activity by storage under H_2 at 0° for 24 hr.

An experiment was conducted to determine the influence of different gases on reactivation of the cold inactivated enzyme complex. The initial extract fixed N_2^{15} to give 0.787 atom per cent N^{15} excess in an hour, but after storage at 0° for 24 hr its fixation was only 0.072 atom per cent N^{15} excess. Samples of the partially inactivated extract were incubated for an hour at 22° under H_2, N_2, and argon, and then in an hour under N_2^{15} they fixed 0.381, 0.030, and 0.091 atom per cent N^{15} excess, respectively. It is apparent that there was substantial reactivation only under H_2.

H_2 affords some protection during storage at 0°. Extracts stored for 24 hr under H_2 retained 4.4 per cent of their activity and could be restored to 34.5 per cent of

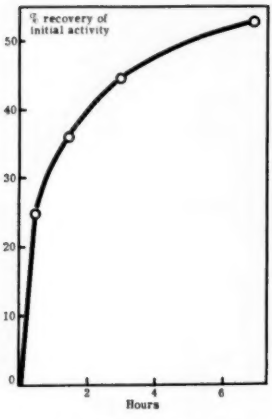

FIG. 6.—Time course of reactivation at 22° under H_2 of the N_2-fixing enzyme complex which had been inactivated by storage under H_2 at 0° for 24 hr.

their initial activity in an hour under H_2 at 20°. In contrast, extracts stored under N_2 or argon lost their activity, and only 5.9 per cent and 4.3–9.2 per cent, respectively, of their initial activity was restored by incubation for an hour under H_2. Prolonging incubation of the argon-stored extract to 5 hr under H_2 restored 30 per cent of the initial activity. Apparently H_2 is specifically functional in the reactivation.

Extracts from *C. pasteurianum* inactivated by storage at 0° for 24 hr were treated with 6 *M* urea for 15 min to effect an unfolding of the protein. Urea was removed by dialysis in the cold or at room temperature, and capacity for N_2 fixation was measured in the presence of boiled extract to furnish any cofactors lost by dialysis. The extracts did not recover their activity. Cold inactivated extracts were adjusted to pH 4 or pH 8 and were held for 10 min before readjusting to pH 6.5 for assay of their activity. These treatments restored no N_2-fixing activity. Although cold inactivation possibly results from a folding or from an aggregation of protein to mask its active sites, the possibility is not supported by the facts that dilute extracts are more cold labile than concentrated extracts or that attempts to unfold the cold inactivated protein have restored no activity. Definitive studies to establish the mode of cold inactivation probably must be deferred until a highly purified enzyme can be prepared.

Summary.—Extracts from *Clostridium pasteurianum* lose approximately 95 per cent of their activity for N_2 fixation during storage under H_2 at 0° for 24 hr. Among the stabilizing agents tested, ascorbic acid, $Na_2S_2O_4$, and reduced lipoic acid were helpful. The N_2-fixing enzyme complex is cold labile and retains activity best near 20° when stored under H_2. Preparations partially inactivated by storage for 6 hr at 0° were restored to full activity by incubation under H_2 at 22° for an hour, whereas preparations stored for 24 hr at 0° recovered only 57 per cent of their activity under H_2 at 22° for 7 hr. H_2 is specifically functional in reactivation, but the basis for inactivation and reactivation of the N_2-fixing complex is not apparent.

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*SEPARATION OF THE BIOSYNTHESIS OF THE ANTIBIOTIC POLYPEPTIDE TYROCIDINE FROM PROTEIN BIOSYNTHESIS**

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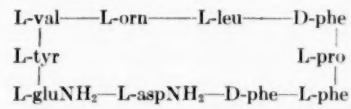
Recent findings have clarified many of the enzymatic processes which are required for the incorporation of amino acids into protein,[1] and the biosynthesis of protein is now known to differ from that of glutathione[2] and from that of the peptides of bacterial cell wall nucleotides[3] or of actinomycin.[4] Spore-forming bacteria, at a certain stage of their life cycle, produce large amounts of polypeptide entities whose molecular weights extend over a wide range, from gramicidin J (mol. wt. 800),[5] to tyrocidine (mol. wt. 1,300),[6] subtilin (mol. wt. 3,300),[7] lichenformin (mol. wt. 4,000),[8] and the proteins of the clostridial toxins[9] and of the crystals of *B. thurigiensis*.[10] The biosynthetic mechanisms involved in the formation of most of these molecules, like that of some pituitary hormones (mol. wt. 1,000),[11] remain obscure. The polypeptides of this group could be synthesized along a ribosomal template as are proteins; they could result from the breakdown of larger protein molecules; or they might be synthesized without an RNA template by the stepwise enzymatic addition of amino acids.

Tyrocidine, produced by *Bacillus brevis*,[12, 13] is a cyclic decapeptide with the following amino acid sequence:[14]



Tyrocidine has been selected for this study because: (1) its amino acids, except for ornithine, are shared with protein but are not known constituents of bacterial cell wall peptides; (2) the amino acid sequence of the polypeptide has been determined, and single amino acid differences have been described between tyrocidines A, B, and C;[14, 15] (3) the solubility properties and stability of tyrocidine make its isolation and purification relatively simple.

Existing information about the biosynthesis of these polypeptides has resulted from long-term experiments during which any modification of protein synthesis or of protein structure might indirectly affect other biosynthetic processes.[16] For example, in a study of the effects of various metabolic inhibitors on the biosynthesis of gramicidin S, the observed changes could not be assigned to primary or secondary actions of these compounds.[16] We have employed an experimental design which makes possible the study of tyrocidine and protein synthesis over very short time periods. It was thus possible to investigate the two processes independently of

each other. The data reported in this paper indicate that the biosynthesis of this polypeptide is a process distinct from the biosynthesis of proteins.

Materials and Methods.—Bacillus brevis: A single rough colony, which had been isolated from ATCC 10068, was selected for further study on the basis of its stable colony morphology, reproducible sporulation, and infrequency of mutation to smooth variants. This strain produced high levels of antibiotic activity, and the tyrocidine isolated from the cultures was shown[17] to be identical in amino acid composition with that previously described.

Micrococcus conglomeratus was supplied by J. L. Stokes.

Asparagine-glycerol minimal medium (AGMM) was modified from Stokes and Woodward[18] and contained per 1,000 ml H_2O: K_2HPO_4, 0.5 gm; KH_2PO_4, 0.5 gm; $MgSO_4 \cdot 7H_2O$, 0.2 gm; NaCl, 0.01 gm; $MnSO_4 \cdot 4H_2O$, 0.01 gm; $FeSO_4 \cdot 7H_2O$, 0.01 gm; $CaH_4(PO_4)_2$ H_2O sat. sol., 2 ml; L-asparagine, 2.5 gm; glycerol, 4 ml.

Acid alumina was prepared from neutral alumina (Bio-Rad Laboratories) according to Wieland.[19] Commercial dimethylaminobenzaldehyde (DMAB) was recrystallized according to Adams and Coleman.[20] L-tyrosine-U-C^{14} and D,L-ornithine-2-C^{14} were obtained, respectively, from Nuclear-Chicago and from California Corporation for Biochemical Research. L-proline-3, 4-H^3 and L-aspartic acid-U-C^{14} were obtained from New England Nuclear Corporation and $H_2S^{35}O_4$ from Oak Ridge National Laboratory. We thank Dr. K. Pfister (Merck, Sharp and Dohme), Dr. H. Snyder (Dept. of Chemistry, University of Illinois), Dr. W. Shive (Dept. of Chemistry, University of Texas) and Dr. R. Schaefer (Lederle Laboratories) for gifts of α-methyltryptophan, β-methyltryptophan, methallyl-glycine, and chlortetracycline HCl (aureomycin), respectively. All other compounds were acquired from regular commercial sources.

Bioassay of tyrothricin (the naturally occurring mixture of tyrocidine and gramicidin): Five ml of *B. brevis* cultures grown at 37° with aeration by rotary shaking were added to 25 ml of absolute alcohol and allowed to stand 24 hr. After centrifugation the antibiotic potency of the resulting extract was assayed by serial dilutions and compared with the activity of a commercial sample of tyrothricin. The assay consisted of 2 ml of medium (1% yeast extract, 0.25% glycerol) inoculated with exponentially growing *M. conglomeratus* and to which an aliquot of the tyrothricin extract was added. Bacterial growth was measured by O. D. at 600 mμ in a Zeiss spectrophotometer.

Bioautography: After separation by paper electrophoresis (see below), gramicidin and tyrocidine were located on the basis of their antibiotic activity. The paper strip was placed in contact with sterile agar medium (1% yeast extract, 0.25% glycerol, 1.5% agar) for 15 min. After removal of the paper, the agar was sprayed with a culture of *M. conglomeratus* and incubated overnight at 37°. Distinct zones of growth inhibition indicated antibiotic activity.

Incorporation of C^{14} amino acids into tyrocidine: Five hundred ml of AGMM were inoculated with *B. brevis* spores (5×10^8/ml) and incubated at 37° on a rotary shaker. When the O. D. (600 mμ) of the culture had reached a value of 1.8–2.0, 10 ml aliquots were transferred to separate flasks. L-tyrosine-C^{14} or D,L-ornithine-C^{14} (final concentration: 1 μc/μmole, 0.25 μC/ml) were added, and the incubation was continued. At time zero and at later times as indicated, the experiment was terminated by the addition of nonradioactive tyrosine, or ornithine, and 50 ml of ethanol. Carrier tyrocidine (1 mg) was added to each vessel, and isolated as described below. Radioactivity of tyrocidine was expressed as counts/min/mg of tyrocidine recovered.

Extraction of tyrothricin: After extraction in ethanol for 24 hr, the samples were centrifuged and dried by flash evaporation. The residues were dissolved in 3 ml of 90% ethanol and passed through a column (4.5×1.5 cm) of acid alumina. It was observed that, as described by Wieland,[21] all free neutral and basic amino acids adsorbed to the alumina, but that under these conditions more than 95% of the tyrothricin emerged with the solvent front.

Separation of tyrocidine and gramicidin: The effluent from the alumina column was taken to dryness, dissolved in a small volume of methanol buffer (methanol, acetic acid, water, 3:1:1), pH 2.5, and an aliquot of the solution applied to a strip of Whatman 31 paper. Tyrocidine was separated from gramicidin by electrophoresis in an electric field of 16 volts/cm in the same methanol buffer. As shown on Figure 1, gramicidin stayed close to the origin, whereas tyrocidine moved toward the anode due to its basic ornithine residue. This separation was confirmed with authentic samples of tyrocidine and gramicidin which had been isolated by countercurrent distribution.[22] A small strip of paper was cut off and stained with diazotized sulfanilic acid[23] to

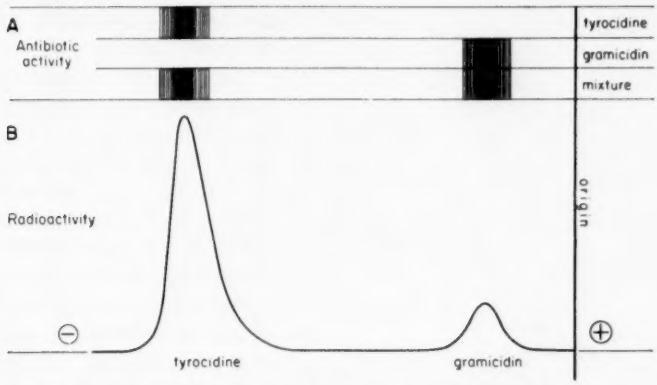


FIG. 1.—Separation of tyrocidine and gramicidin by paper electrophoresis. Under the condition described in *Methods*, tyrocidine and gramicidin moved 8 and 2 inches, respectively, toward the anode in 10 hr. (*A*) Pure samples of the two compounds were applied at the origin and, after electrophoresis, were localized by bioautography. (*B*) Tyrocidine and gramicidin were also separated from tyrothricin isolated after incubation of *B. brevis* cultures with C^{14}-tyrosine. Radioactivity was located on the paper in a Nuclear-Chicago scanner.

locate tyrocidine. The antibiotic was then eluted in methanol buffer from the corresponding zone of the rest of the paper. Samples isolated by this method were shown[24] to be composed of the mixture of tyrocidines A, B, and C known to constitute tyrocidine. The method was reproducible to within 5% and the total yield of tyrocidine was 92–94%.

Specific activity of isolated tyrocidine: Tyrocidine was measured by a modification of the dimethylaminobenzaldehyde reaction.[25] Aliquots of the samples in methanol buffer were mixed with 0.5 ml of a 5% DMAB solution in conc. HCl. One ml of HCl was added, followed 5 min later by 0.1 ml of 0.1% sodium nitrite, and 1 ml of H_2O. After 20 min the O.D. of each sample was measured at 575 mμ. The standard curve was linear between 20 and 200 μg of tyrocidine. Radioactivity was determined on an aliquot of the tyrocidine eluate in a Tri-Carb liquid scintillation spectrometer.

Incorporation of methionine-S^{35} into protein: After incubation with methionine-S^{35} (0.5 μC/ml, 1 μC/μmole) or with $S^{35}O_4^{--}$ (5μC/ml), aliquots of a *B. brevis* culture were chilled in 0.5 *N* $HClO_4$. Following a 30 min incubation at 90°, the samples were washed three times with 0.25 *N* $HClO_4$, once with ethanol-ether (1:3), and with ether, and then dissolved in 1 *N* NaOH. Aliquots were used for estimation of radioactivity and for measurement of protein by the Lowry method.[26]

Results.—(1) *Production of tyrocidine by shake cultures of Bacillus brevis:* Under the conditions described in *Methods*, tyrothricin and tyrocidine are produced at a rapid rate for a short period of time (Fig. 2). The onset of synthesis occurs reproducibly approximately 26 hr after inoculation, at which time the optical density (600 mμ) of the culture is 1.2, and the most rapid rate is achieved at an O.D. of 2. This corresponds to a bacterial dry weight of 3.3 mg/ml. The production of antibiotic follows the completion of the exponential phase of growth and precedes visible sporulation, as was found also for bacitracin production by *B. lichenformis* cultures.[27]

During this period of maximal antibiotic production, the incorporation of C^{14} amino acid precursors into tyrocidine may be used as a measure of polypeptide biosynthesis. Incorporation is linear with time for at least 20 min, during which time tyrosine can be incorporated into tyrocidine at a rate of 0.1 mμmole/min/ml of culture; this corresponds to more than half the rate of incorporation of tyrosine into all proteins. This rate of synthesis permits observations to be made for periods of a few minutes so that the direct effect of various agents on the process of

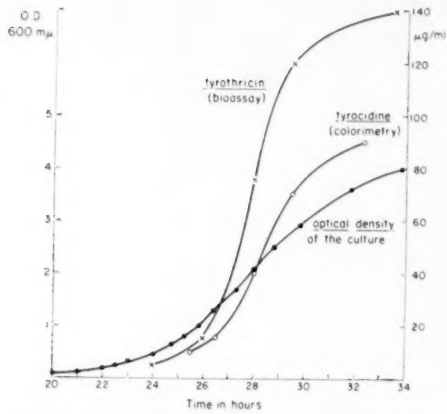


FIG. 2.—The production of tyrocidine by a culture of *Bacillus brevis*. The optical density of the culture grown as described in *Methods* was measured at 600 mμ. Tyrothricin and tyrocidine were measured in aliquots of the culture by bioassay and colorimetry, respectively.

polypeptide formation can be studied.

(2) *The effect of chloramphenicol, puromycin, and aureomycin on the biosynthesis of tyrocidine:* The data in Figures 3 and 4 show that incorporation of S^{35} amino acids into protein of *B. brevis* cultures is sensitive to low concentrations of the three antibiotics tested. In contrast, tyrosine-C^{14} incorporation into tyrocidine is immune to much higher concentrations of chloramphenicol and puromycin. In similar experiments, the incorporation of ornithine-C^{14}, proline-H^3, and aspartic acid-C^{14} into tyrocidine was also insensitive to chloramphenicol. At the low concentrations of aureomycin which suppress protein synthesis almost completely, tyrocidine production is unaffected (Fig. 4), although higher levels of this antibiotic inhibit polypeptide formation. Since aureomycin probably interferes with more than one physiological function, this finding is not unexpected.

(3) *Effect of various amino acid analogues on protein and tyrocidine biosynthesis:* The effects of a number of amino acid analogues on the synthesis of protein and tyrocidine by *B. brevis* have been compared in the hope of further differentiating these processes. The action of these compounds on tyrocidine formation was studied in the presence of chloramphenicol to exclude any secondary effects which might result from analogue incorporation into the enzyme systems responsible for antibiotic production. Protein synthesis was measured in parallel samples in the absence of chloramphenicol. From the data in Figure 5 it can be seen that several amino acid analogues inhibited tyrocidine biosynthesis without altering that of protein. Conversely, other analogues affected the biosynthesis of protein and not that of the antibiotic. The effects of *m*- and *o*-fluorophenylalanine were identical with that of *p*-fluorophenylalanine, and the effect of erythro-β-phenylserine was identical with that of its threo isomer. However, β-methyltryptophan did not inhibit tyrocidine synthesis, whereas α-methyltryptophan did.

(4) *Attempts to isolate mutant strains of B. brevis:* Attempts to isolate mutant strains of *B. brevis* incapable of synthesizing specific amino acids have so far failed. However, several strains have been obtained which do not produce detectable amounts of antibiotics.

Discussion.—The results of the experiments described above make it appear unlikely that tyrocidine biosynthesis shares many of the enzymatic steps which have been demonstrated for protein synthesis.

Chloramphenicol and puromycin are known to interfere with the transfer of amino acids from sRNA into peptide linkages at the ribosomal sites which function in protein synthesis.[28] If the biosynthesis of tyrocidine involved a similar step, a

corresponding susceptibility to chloramphenicol and puromycin might be expected. Since none was found, a transfer step resembling that known for protein synthesis, and involving aminoacyl sRNA as a precursor, is unlikely to be involved in tyrocidine formation. The incorporation into tyrocidine, in the absence of protein synthesis, of four radioactive amino acids whose residues are scattered throughout the tyrocidine molecule also shows that the entire amino acid sequence of the antibiotic is not merely a polypeptide fragment derived from pre-existing protein.

The failure of chloramphenicol and puromycin to stimulate the formation of tyrocidine suggests that the availability of free amino acids is not a rate-limiting factor in the biosynthesis of the polypeptide, whereas it appears to be in actinomycin production.[29] The decreased rate of amino acid incorporation seen after 20 min in the presence of these antibiotics might result from the decay of an essential enzyme which could not be regenerated in their presence, or from the termination in some other way of the phase of the bacterial life cycle during which polypeptide synthesis takes place.

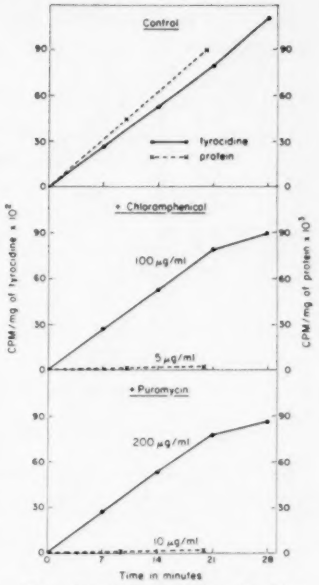


FIG. 3.—The effect of chloramphenicol and puromycin on the biosynthesis of tyrocidine and of protein. Incorporation of C^{14}-tyrosine into tyrocidine and of methionine-S^{35} into protein was measured as described in *Methods*. Chloramphenicol or puromycin were added two min before the radioactive amino acid. The points are averages of duplicate experiments, and all results are corrected for 0 time values.

The initial event in the biosynthesis of protein is the recognition and activation of amino acids which precede aminoacylation of specific sRNA molecules by the corresponding activating enzymes. It is known that certain analogues of naturally occurring amino acids interfere with protein synthesis by competing with their normal counterparts at the level of the activating enzymes.[30] Since peptide bond formation is an endothermic reaction, some activation step is presumably required for tyrocidine synthesis, as it is for peptide bond synthesis in protein. Our data show that several amino acid analogues inhibit tyrocidine production without affecting protein synthesis. Although the mechanism of this inhibition is still undefined, it could involve competition for the activation of free amino acids or interference with the activation of growing peptide chains following incorporation of an amino acid analogue. In either case, at least one activation step for tyrocidine synthesis must be enzymatically distinct from that for protein synthesis, since the latter process is unaffected by these compounds. The differential inhibitory spectra, for protein and polypeptide synthesis, of the other amino acid analogues tested also support the conclusion that these two processes must differ significantly in their enzymatic pathways.

The results of another series of experiments[24] on the biosynthesis of tyrocidines A, B, and C led to similar conclusions. They suggest a low level of specificity in the enzymatic mechanisms involved in the recognition and activation of phenylalanine

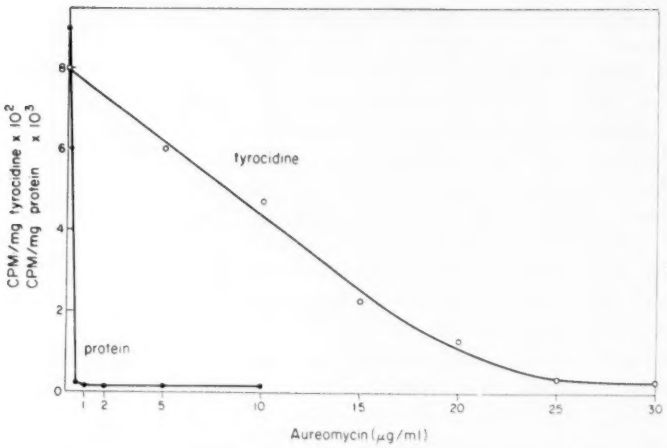


FIG. 4.—The effect of aureomycin on the biosynthesis of tyrocidine and of protein. The conditions were those described for Fig. 3. Aureomycin, at the concentration indicated, was added 2 min before the radioactive precursor.

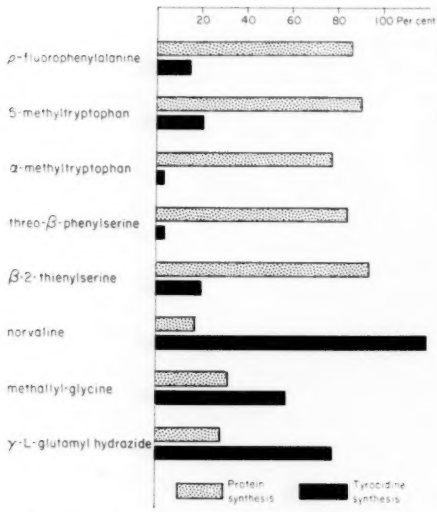


FIG. 5.—The effect of some amino acid analogues on the biosynthesis of tyrocidine and of protein. Incorporation of D,L-ornithine-C^{14} into tyrocidine and of $S^{35}O_4^{--}$ into protein were measured as described in *Methods*. The amino acid analogues (final concentration 200 μg/ml) were added two min before the radioactive precursors. Incorporation of ornithine-C^{14} into tyrocidine was measued in the presence of chloramphenicol (50 μg/ml) which was added 2 min prior to the analoguesl. Incorporation was measured for 20 min and all results are expressed as per cent of the control sample.

and tryptophan; this contrasts with the rigorous specificity exhibited by *B. brevis* extracts in the formation of aminoacyl sRNA with the same two amino acids.[31]

On the basis of the data presented it is concluded that the precise sequential ordering of amino acids in the biosynthesis of tyrocidine proceeds by a process fundamentally different from that characterized for protein synthesis. The existence of another entire set of specific transfer-RNA molecules whose function is restricted to polypeptide synthesis is conceivable; however, simpler mechanisms can be envisaged for the synthesis of these polypeptides. It is possible that specific enzymes, perhaps a multienzyme complex such as functions in the synthesis of fatty acids,[32] could embody the structural specificity required for the recognition of both the unfinished peptide chain and the next free amino acid, and thus direct the ordered stepwise addition of amino acids. Other polypeptides may be

synthesized like tyrocidine rather than like protein, and it would be of interest to establish what factors determine the selection of one or the other peptide-forming system for the biosynthesis of polypeptides.

Summary.—The biosyntheses of tyrocidine and of protein by *B. brevis* have been compared. Several compounds were found to affect the two processes independently, indicating distinct differences between the two types of syntheses.

Chloramphenicol and puromycin effectively inhibited protein synthesis but had no effect on the formation of tyrocidine.

Several analogues of amino acids inhibited the biosynthesis of tyrocidine without affecting that of protein. Other amino acid analogues inhibited the production of protein but not of tyrocidine.

It is concluded that tyrocidine is synthesized by mechanisms different from those involved in the biosynthesis of protein, and it is suggested that this may be the case for other polypeptides of this size group.

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Newman, Melvin Spencer, 1956 (5), 2239 Onandaga Drive, Columbus 21, Ohio

Neyman, Jerzy, 1963 (1), Department of Statistics, University of California, Berkeley 4, California

Nicholas, John Spangler, 1949 (8), Osborn Zoological Laboratory, Yale University, New Haven 11, Connecticut

Nicholson, Seth Barnes, 1937 (2), Mount Wilson and Palomar Observatories, 813 Santa Barbara Street, Pasadena, California

Niemann, Carl George, 1952 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Nier, Alfred Otto C., 1950 (3), School of Physics, University of Minnesota, Minneapolis 14, Minnesota

Nolan, Thomas Brennan, 1951 (6), 2219 California Street, N. W., Washington 8, D. C.

Northrop, John Howard, 1934 (14), Department of Bacteriology, University of California, Berkeley 4, California

Noyes, William Albert, Jr., 1943 (5), Department of Chemistry, University of Rochester, Rochester 20, New York. After September 1, 1963: Department of Chemistry, University of Texas, Austin 12, Texas

O'Brien, Brian, 1954 (3), Box 117, Pomfret, Connecticut

Ochoa, Severo, 1957 (14), New York University School of Medicine, 550 First Avenue, New York 16, New York

Olson, Harry Ferdinand, 1959 (4), RCA Laboratories, David Sarnoff Research Center, Princeton, New Jersey

Oncley, John Lawrence, 1947 (14), Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, Michigan

Onsager, Lars, 1947 (5), Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut

Opie, Eugene Lindsay, 1923 (10), Rockefeller Institute, New York 21, New York

Oppenheimer, J. Robert, 1941 (3), The Institute for Advanced Study, Princeton, New Jersey

Osterhout, Winthrop John Vanleuven, 1919 (7), 450 East 63rd Street, New York 21, New York

Painter, Theophilus Shickel, 1938 (8), University of Texas, Austin 12, Texas

Pais, Abraham, 1962 (3), Rockefeller Institute, New York 21, New York

Palade, George Emil, 1961 (8), Rockefeller Institute, New York 21, New York

Panofsky, Wolfgang **K. H.,** 1954 (3), Stanford Linear Accelerator Center, Stanford University, Stanford, California

Patterson, Bryan, 1963 (6), Museum of Comparative Zoology at Harvard College, Oxford Street, Cambridge, 38, Massachusetts

Paul, John Rodman, 1945 (10), Yale University School of Medicine, 333 Cedar Street, New Haven 11, Connecticut

Pauling, Linus, 1933 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California

Pekeris, Chaim Leib, 1952 (13), Department of Applied Mathematics, The Weizmann Institute of Science, Rehovot, Israel

Perlman, Isadore, 1963 (5), Department of Chemistry, University of California, Berkeley 4, California

Petrunkevitch, Alexander, 1954 (8), Osborn Zoological Laboratory, Yale University, New Haven 11, Connecticut

Pfaffmann, Carl, 1959 (12), Walter S. Hunter Laboratory of Psychology, Brown University, Providence 12, Rhode Island

Pickering, William Hayward, 1962 (4), Jet Propulsion Laboratory, 4800 Oak Grove Drive, Pasadena, California

Pierce, John Robinson, 1955 (4), Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey

Piggot, Charles Snowden, 1946 (13), Holly Grove, Route 2, Box 68, Lexington Park, Maryland

Piore, Emanuel Ruben, 1963 (4), International Business Machines Corporation, 590 Madison Avenue, New York 22, New York

Pittendrigh, Colin Stephenson, 1963 (8), Department of Biology, Princeton University, P.O. Box 704, Princeton, New Jersey

Pitts, Robert Franklin, 1956 (9), Cornell University Medical College, 1300 York Avenue, New York 21, New York

Pitzer, Kenneth Sanborn, 1949 (5), Rice University, Houston 1, Texas

Pound, Robert Vivian, 1961 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

Press, Frank, 1958 (13), Seismological Laboratory, California Institute of Technology, 220 North San Rafael Avenue, Pasadena, California

Puck, Theodore Thomas, 1960 (10), Department of Biophysics, University of Colorado Medical Center, Denver 20, Colorado

Purcell, Edward Mills, 1951 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

Rabi, Isidor Isaac, 1940 (3), Department of Physics, Columbia University, New York 27, New York

Ramsey, Norman Foster, 1952 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

Raper, Kenneth Bryan, 1949 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Raymond, Arthur Emmons, 1950 (4), 73 Oakmont Drive, Los Angeles 49, California

Redfield, Alfred Clarence, 1958 (13), P.O. Box 106, Woods Hole, Massachusetts

Reichelderfer, Francis Wilton, 1945 (13), United States Weather Bureau, Department of Commerce, Washington 25, D. C.

Revelle, Roger Randall, 1957 (13), 714 University Hall, University of California, Berkeley 4, California

Rhoades, Marcus Morton, 1946 (7), Department of Botany, Indiana University, Bloomington, Indiana

Rich, Arnold Rice, 1954 (10), Johns Hopkins Hospital, Baltimore 5, Maryland

Richards, Alfred Newton, 1927 (9), 737 Rugby Road, Bryn Mawr, Pennsylvania

Richards, Dickinson W., 1958 (9), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

Richter, Curt Paul, 1948 (12), Johns Hopkins Hospital, Baltimore 5, Maryland

Riddle, Oscar, 1939 (8), Route 4, Plant City, Florida

Riggs, Lorrin Andrews, 1961 (12), Hunter Laboratory of Psychology, Brown University, Providence 12, Rhode Island

Riker, Albert Joyce, 1951 (7), Department of Plant Pathology, University of Wisconsin, Madison 6, Wisconsin

Rittenberg, David, 1953 (14), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

Robbins, William Jacob, 1940 (7), 15 Dellwood Circle, Bronxville, New York

Roberts, John D., 1956 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Roberts, Richard Brooke, 1961 (7), Department of Terrestrial Magnetism, Carnegie Institution of Washington, 5241 Broad Branch Road, N. W., Washington 15, D. C.

Robertson, Oswald Hope, 1943 (10), 9150 Los Gatos Highway, Santa Cruz, California

Romer, Alfred Sherwood, 1944 (8), Museum of Comparative Zoology at Harvard College, Oxford Street, Cambridge 38, Massachusetts

Rose, William Cumming, 1936 (14), University of Illinois, Urbana, Illinois

Rossi, Bruno Benedetto, 1950 (3), Room 26–569, Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Rossini, Frederick Dominic, 1951 (5), University of Notre Dame, Notre Dame, Indiana

Rous, Francis **Peyton,** 1927 (10), Rockefeller Institute, New York 21, New York

Rouse, Irving, 1962 (11), Department of Anthropology, Yale University, Box 2114, Yale Station, New Haven, Connecticut

Rubey, William Walden, 1945 (6), Department of Geology and Institute of Geophysics, University of California, Los Angeles 24, California

Russell, Richard Joel, 1959 (6), Coastal Studies Institute, Louisiana State University, Baton Rouge 3, Louisiana

Sabin, Albert Bruce, 1951 (10), The Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Elland Avenue and Bethesda, Cincinnati 29, Ohio

Sandage, Allan Rex, 1963 (2), Mount Wilson and Palomar Observatories, 813 Santa Barbara Street, Pasadena, California

Savage, John Lucian, 1949 (4), 1651 Dahlia Street, Denver 20, Colorado

Sax, Karl, 1941 (7), Bishop Hollow Road, Media, Pennsylvania

Scatchard, George, 1946 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Schairer, John Frank, 1953 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

Schiff, Leonard Isaac, 1957 (3), Department of Physics, Stanford University, Stanford, California

Schmidt, Carl Frederic, 1949 (9), Aviation Medical Acceleration Laboratory, United States Naval Air Development Center, Johnsville, Pennsylvania

Schmidt-Nielsen, Knut, 1963 (8), Department of Zoology, Duke University, Durham, North Carolina

Schmitt, Francis Otto, 1948 (8), Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Scholander, Per Fredrik, 1961 (8), Scripps Institution of Oceanography, University of California, La Jolla, California

Schwarzschild, Martin, 1956 (2), Princeton University Observatory, 265 Fitz Randolph Road, Princeton, New Jersey

Schwinger, Julian, 1949 (3), Department of Physics, Harvard University, Cambridge 38, Massachusetts

Seaborg, Glenn Theodore, 1948 (5), United States Atomic Energy Commission, Washington 25, D. C.

Seares, Frederick Hanley, 1919 (2), Halekulani Hotel, Honolulu 15, Hawaii

Segrè, Emilio, 1952 (3), Department of Physics, University of California, Berkeley 4, California

Seitz, Frederick, 1951 (3), Department of Physics, University of Illinois, Urbana, Illinois

Serber, Robert, 1952 (3), Department of Physics, Columbia University, New York 27, New York

Shane, Charles **D**onald, 1961 (2), P.O. Box 582, Santa Cruz, California

Shannon, Claude Elwood, 1956 (1), Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shapiro, Harry **L**ionel, 1949 (11), American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

Shapley, Harlow, 1924 (2), Sharon Cross Road, Peterboro, New Hampshire

Shedlovsky, Theodore, 1953 (5), Rockefeller Institute, New York 21, New York

Sheehan, John Clark, 1957 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shemin, David, 1958 (14), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

Sherwood, Thomas Kilgore, 1958 (4), Room 12-186, Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Shockley, William, 1951 (4), Shockley Laboratory, Clevite Transistor, 1801 Page Mill Road, Palo Alto, California

Shope, Richard Edwin, 1940 (10), Rockefeller Institute, New York 21, New York

Simpson, George **G**aylord, 1941 (6), Museum of Comparative Zoology at Harvard College, Cambridge 38, Massachusetts

Simpson, John Alexander, 1959 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

Sinnott, Edmund Ware, 1936 (7), Osborn Biological Laboratory, Yale University New Haven, Connecticut

Skinner, Burrhus **F**rederic, 1950 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

Skoog, Folke Karl, 1956 (7), Department of Botany, Birge Hall, University of Wisconsin, Madison 6, Wisconsin

Slater, John Clarke, 1932 (3), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Slepian, Joseph, 1941 (4), 1115 Lancaster Street, Pittsburgh 18, Pennsylvania

Slichter, Louis Byrne, 1944 (13), Institute of Geophysics, University of California, Los Angeles 24, California

Slipher, Vesto **M**elvin, 1921 (2), P.O. Box 326, Flagstaff, Arizona

Smadel, Joseph Edwin, 1957 (10), Laboratory of Virology and Rickettsiology, Division of Biologics Standards, National Institutes of Health, Bethesda 14, Maryland

Smith, Albert Charles, 1963 (7), Smithsonian Institution, Washington 25, D. C.

Smith, Cyril Stanley, 1957 (4), Room 14N–317, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Smith, Emil L., 1962 (14), Department of Biological Chemistry, University of California Medical Center, Los Angeles 24, California

Smith, Lee Irvin, 1944 (5), School of Chemistry, University of Minnesota, Minneapolis 14, Minnesota

Smith, Paul Althaus, 1947 (1), Department of Mathematics, Columbia University, New York 27, New York

Smith, Philip Edward, 1939 (8), Department of Anatomy, Stanford University, Stanford, California

Smyth, Charles Phelps, 1955 (5), Department of Chemistry, Princeton University, Princeton, New Jersey

Snell, Esmond Emerson, 1955 (14), Department of Biochemistry, University of California, Berkeley 4, California

Soderberg, Carl Richard, 1947 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Sonneborn, Tracy Morton, 1946 (8), 220 Jordan Hall, Indiana University, Bloomington, Indiana

Spedding, Frank Harold, 1952 (5), Box 14-A, University Station, Institute for Atomic Research, Iowa State University, Ames, Iowa

Spence, Kenneth Wartinbe, 1955 (12), Department of Psychology, State University of Iowa, Iowa City, Iowa

Spencer, Donald Clayton, 1961 (1), Fine Hall, Princeton University, P. O. Box 708, Princeton, New Jersey

Sperry, Roger Wolcott, 1960 (12), Division of Biology, California Institute of Technology, Pasadena, California

Spitzer, Lyman, Jr., 1952 (2), Princeton University Observatory, 265 FitzRandolph Road, Princeton, New Jersey

Sporn, Philip, 1962 (4), American Electric Power Company, Inc., 2 Broadway, New York 8, New York

Stakman, Elvin Charles, 1934 (7), Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota

Stanley, Wendell Meredith, 1941 (14), Virus Laboratory, University of California, Berkeley 4, California

Stebbins, George Ledyard, 1952 (7), Department of Genetics, University of California, Davis, California

Stebbins, Joel, 1920 (2), 69 University Drive, Menlo Park, California

Steenrod, Norman Earl, 1956 (1), Fine Hall, Princeton University, P. O. Box 708, Princeton, New Jersey

Stein, William Howard, 1960 (14), Rockefeller Institute, New York 21, New York

Stern, Curt, 1948 (8), Department of Zoology, University of California, Berkeley 4, California

Stern, Otto, 1945 (3), 759 Cragmont Avenue, Berkeley 8, California

Stevens, Stanley Smith, 1946 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

Steward, Julian H., 1954 (11), Department of Anthropology, 137 Davenport Hall, University of Illinois, Urbana, Illinois

Stewart, Thomas Dale, 1962 (11), Museum of Natural History, Smithsonian Institution, Washington 25, D. C.

Stockmayer, Walter Hugo, 1956 (5), Department of Chemistry, Dartmouth College, Hanover, New Hampshire

Stoker, James Johnston, 1963 (1), New York University, 4 Washington Place, New York 3, New York

Stommel, Henry Melson, 1961 (13), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Stone, Marshall Harvey, 1938 (1), 303 Eckhart Hall, University of Chicago, Chicago 37, Illinois

Stone, Wilson Stuart, 1960 (8), Genetics Foundation, University of Texas, Austin 12, Texas

Stork, Gilbert Josse, 1960 (5), Department of Chemistry, Columbia University, New York 27, New York

Stratton, Julius Adams, 1950 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Straus, William Louis, Jr., 1962 (8), Department of Anatomy, School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore 5, Maryland

Street, Jabez Curry, 1953 (3), Jefferson Physical Laboratory, Harvard University, Cambridge 38, Massachusetts

Sturtevant, Alfred Henry, 1930 (8), California Institute of Technology, Pasadena 4, California

Suits, Chauncey Guy, 1946 (4), General Electric Research Laboratory, The Knolls, Schenectady, New York

Szent-Györgyi, Albert, 1956 (14), Institute for Muscle Research, P. O. Box 187, Woods Hole, Massachusetts

Szilard, Leo, 1961 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

Taliaferro, William Hay, 1940 (10), Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois

Tarbell, Dean Stanley, 1959 (5), Department of Chemistry, University of Rochester, Rochester 20, New York

Tatum, Edward Lawrie, 1952 (14), Rockefeller Institute, New York 21, New York

Taube, Henry, 1959 (5), Department of Chemistry, Stanford University, Stanford, California

Teller, Edward, 1948 (3), Lawrence Radiation Laboratory, University of California, Berkeley 4, California

Terman, Frederick Emmons, 1946 (4), Provost's Office, Stanford University, Stanford, California

Thimann, Kenneth Vivian, 1948 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Thomas, Charles Allen, 1948 (4), Monsanto Chemical Company, 800 North Lindbergh Boulevard, St. Louis 66, Missouri

Thomas, Llewellyn Hilleth, 1958 (3), Watson Scientific Laboratory, 612 West 115th Street, New York 25, New York

Thomas, Tracy Yerkes, 1941 (1), Department of Mathematics, Indiana University, Bloomington, Indiana

Tillett, William Smith, 1951 (10), New York University School of Medicine, 550 First Avenue, New York 16, New York

Timoshenko, Stephen Prokop, 1940 (4), 536 West Crescent Drive, Palo Alto, California

Tishler, Max, 1953 (5), Merck & Co., Inc., Rahway, New Jersey

Tousey, Richard, 1960 (2), United States Naval Research Laboratory, Washington 25, D. C.

Townes, Charles Hard, 1956 (3), Provost's Office, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Tukey, John Wilder, 1961 (1), Fine Hall, Princeton University, P. O. Box 708, Princeton, New Jersey

Turner, Francis John, 1956 (6), Department of Geology and Geophysics, University of California, Berkeley 4, California

Tuve, Merle Antony, 1946 (3), Department of Terrestrial Magnetism, Carnegie Institution of Washington, 5241 Broad Branch Road, N. W., Washington 15, D. C.

Twitty, Victor Chandler, 1950 (8), Department of Biological Sciences, Stanford University, Stanford, California

Uhlenbeck, George Eugene, 1955 (3), Rockefeller Institute, New York 21, New York

Urey, Harold Clayton, 1935 (5), University of California, San Diego, La Jolla, California

Van Allen, James Alfred, 1959 (13), Department of Physics and Astronomy, State University of Iowa, Iowa City, Iowa

Van Niel, Cornelis Bernardus, 1945 (7), Hopkins Marine Station of Stanford University, Pacific Grove, California

Van Slyke, Donald Dexter, 1921 (14), Brookhaven National Laboratory, Upton, Long Island, New York

Van Vleck, John Hasbrouck, 1935 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

Verhoogen, John, 1956 (13), Department of Geology, University of California, Berkeley 4, California

Vestine, Ernest Harry, 1954 (13), Rand Corporation, 1700 Main Street, Santa Monica, California

Vickery, Hubert Bradford, 1943 (14), Connecticut Agricultural Experiment Station, New Haven 4, Connecticut

Villard, Oswald Garrison, Jr., 1958 (13), Radioscience Laboratory, Stanford University, Stanford, California

Visscher, Maurice Bolks, 1956 (9), Department of Physiology, University of Minnesota, Minneapolis 14, Minnesota

von **Békésy, Georg,** 1956 (12), Psycho-Acoustic Laboratory, Memorial Hall, Harvard University, Cambridge 38, Massachusetts

Waksman, Selman Abraham, 1942 (10), Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

Wald, George, 1950 (14), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Walker, John Charles, 1945 (7), 206 Horticulture Building, University of Wisconsin, Madison 6, Wisconsin

Wall, Frederick Theodore, 1961 (5), Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois

Walsh, Joseph Leonard, 1936 (1), Widener 474, Harvard University, Cambridge 38, Massachusetts

Warner, John Christian, 1956 (5), Carnegie Institute of Technology, Pittsburgh 13, Pennsylvania

Warren, Shields, 1962 (10), Cancer Research Institute, New England Deaconess Hospital, 194 Pilgrim Road, Boston 15, Massachusetts

Washburn, Sherwood Larned, 1963 (11), Department of Anthropology, University of California, Berkeley 4, California

Watson, Cecil James, 1959 (10), Department of Medicine, University of Minnesota Hospital, Minneapolis 14, Minnesota

Watson, James Dewey, 1962 (14), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Webster, David Locke, 1923 (3), 1830 Cowper Street, Palo Alto, California

Weinberg, Alvin Martin, 1961 (3), Oak Ridge National Laboratory, P. O. Box X, Oak Ridge, Tennessee

Weiss, Paul Alfred, 1947 (8), Rockefeller Institute, New York 21, New York

Weisskopf, Victor Frederick, 1952 (3), CERN, Geneva 23, Switzerland

Went, Frits Warmolt, 1947 (7), Missouri Botanical Garden, 2315 Tower Grove Avenue, St. Louis 10, Missouri

Wentzel, Gregor, 1959 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

Westheimer, Frank Henry, 1954 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wetmore, Alexander, 1945 (8), Smithsonian Institution, Washington 25, D. C.

Wetmore, Ralph Hartley, 1954 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Wever, Ernest Glen, 1940 (12), 110 Eno Hall, Princeton University, Princeton, New Jersey

Wheeler, John Archibald, 1952 (3), Palmer Physical Laboratory, Princeton University, Princeton, New Jersey

Whipple, Fred Lawrence, 1959 (2), Astrophysical Observatory, Smithsonian Institution, 60 Garden Street, Cambridge 38, Massachusetts

Whipple, George Hoyt, 1929 (10), School of Medicine and Dentistry, University of Rochester, 260 Crittenden Boulevard, Rochester 20, New York

Whitford, Albert Edward, 1954 (2), Lick Observatory, Mount Hamilton, California

Whitney, Hassler, 1945 (1), The Institute for Advanced Study, Princeton, New Jersey

Whyburn, Gordon Thomas, 1951 (1), Department of Mathematics, University of Virginia, Charlottesville, Virginia

Wick, Gian-Carlo, 1963 (3), Department of Physics, Brookhaven National Laboratory, Upton, Long Island, New York

Wiesner, Jerome Bert, 1960 (4), The White House, Washington, D. C.

Wigner, Eugene Paul, 1945 (3), Fine Hall, Princeton University, Princeton, New Jersey

Wilder, Raymond Louis, 1963 (1), Department of Mathematics, University of Michigan, Ann Arbor, Michigan

Willey, Gordon Randolph, 1960 (11), Peabody Museum, Harvard University, Cambridge 38, Massachusetts

Williams, Carroll Milton, 1960 (8), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

Williams, Howel, 1950 (6), Department of Geology and Geophysics, University of California, Berkeley 4, California

Williams, John Harry, 1961 (3), School of Physics, University of Minnesota, Minneapolis 14, Minnesota

Williams, John Warren, 1952 (5), Department of Chemistry, University of Wisconsin, Madison 6, Wisconsin

Williams, Robert R., 1945 (5), 45 Woodland Avenue, Summit, New Jersey

Williams, Robley Cook, 1955 (14), Virus Laboratory, University of California, Berkeley 4, California

Williams, Roger John, 1946 (14), Clayton Foundation Biochemical Institute, University of Texas, Austin 12, Texas

Willier, Benjamin Harrison, 1945 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, David Wright, 1955 (14), Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Wilson, Edgar Bright, Jr., 1947 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wilson, Edwin Bidwell, 1919 (3), 42 Brington Road, Brookline 46, Massachusetts

Wilson, Olin Chaddock, 1960 (2), Mount Wilson and Palomar Observatories, 813 Santa Barbara Street, Pasadena, California

Wilson, Perry William, 1955 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Wilson, Robert Erastus, 1947 (4), United States Atomic Energy Commission, Washington 25, D. C.

Wilson, Robert Rathbun, 1957 (3), Laboratory of Nuclear Studies, Cornell University, Ithaca, New York

Winstein, Saul, 1955 (5), Department of Chemistry, University of California, Los Angeles 24, California

Wintersteiner, Oskar, 1950 (14), Squibb Institute for Medical Research, New Brunswick, New Jersey

Wolfrom, Melville Lawrence, 1950 (5), Department of Chemistry, The Ohio State University, 88 West 18th Avenue, Columbus 10, Ohio

Wolman, Abel, 1963 (4), 513 Ames Hall, Johns Hopkins University, Baltimore 18, Maryland

Wood, Harland Goff, 1953 (14), Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland 6, Ohio

Wood, William Barry, Jr., 1959 (10), School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore 5, Maryland

Woodring, Wendell Phillips, 1946 (6), United States National Museum, Washington 25, D. C.

Woodward, Robert **B**urns, 1953 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Woolley, Dilworth **W**ayne, 1952 (14), Rockefeller Institute, New York 21, New York

Woolsey, Clinton Nathan, 1960 (9), Laboratory of Neurophysiology, 283 Medical Sciences Building, University of Wisconsin, Madison 6, Wisconsin

Wright, Sewall Green, 1934 (8), Department of Genetics, University of Wisconsin, Madison 6, Wisconsin

Wu, Chien-Shiung, 1958 (3), Department of Physics, Columbia University, New York 27, New York

Wulf, Oliver Reynolds, 1949 (13), United States Weather Bureau, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Wyckoff, Ralph Walter **G**raystone, 1949 (5), Department of Physics, University of Arizona, Tucson 25, Arizona

Yoder, Hatten Schuyler, **Jr.,** 1958 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

Yost, Don Merlin Lee, 1944 (5), California Institute of Technology, Pasadena 4, California

Young, William Gould, 1951 (5), Department of Chemistry, University of California, Los Angeles 24, California

Zacharias, Jerrold Reinach, 1957 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Zachariasen, Frederik **William H**oulder, 1949 (3), Division of the Physical Sciences, University of Chicago, Chicago 37, Illinois

Zariski, Oscar, 1944 (1), Department of Mathematics, Harvard University, 2 Divinity Avenue, Cambridge 38, Massachusetts

Zener, Clarence Melvin, 1959 (4), Westinghouse Research Laboratories, Beulah Road, Churchill Borough, Pittsburgh 35, Pennsylvania

Zimm, Bruno Hasbrouck, 1958 (5), School of Science and Engineering, University of California, San Diego, La Jolla, California

Zinn, Walter **H**enry, 1956 (4), Combustion Engineering, Inc., Nuclear Division, Windsor, Connecticut

Zirkle, Raymond Elliott, 1959 (8), Committee on Biophysics, University of Chicago, 5640 Ellis Avenue, Chicago 37, Illinois

Zworykin, Vladimir **K**osma, 1943 (4), RCA Laboratories, David Sarnoff Research Center, Princeton, New Jersey

Zygmund, Antoni, 1960 (1), Department of Mathematics, University of Chicago, Chicago 37, Illinois

Number of Members July 1, 1963: 673.

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Albright, Fuller, 1952 (10), 271 Goddard Avenue, Brookline 46, Massachusetts

Bailey, Irving **W**idmer, 1929 (7), Harvard University Herbarium, 22 Divinity Avenue, Cambridge 38, Massachusetts

Coble, Arthur Byron, 1924 (1), Lykens Hotel, Lykens, Pennsylvania

Cole, Rufus, 1922 (10), Mt. Kisco, New York

Coolidge, William David, 1925 (3), 1480 Lenox Road, Schenectady 8, New York

Hartman, Carl Gottfried, 1937 (8), 219 Norwood Avenue, North Plainfield, New Jersey

Kelley, Walter Pearson, 1943 (6), 108 Hilgard Hall, University of California, Berkeley 4, California

Schultz, Adolph Hans, 1939 (11), Anthropologisches Institut, Künstlergasse 15, Zurich, Switzerland

Tyzzer, Ernest Edward, 1942 (10), 484 Water Street, Wakefield, Massachusetts

Vandiver, Harry Shultz, 1934 (1), Box 7881, University of Texas, Austin 12, Texas

Number of Members Emeriti July 1, 1963: 10.

FOREIGN ASSOCIATES

The number in parentheses following the year of election indicates association within the Sections of the National Academy of Sciences.

Adrian, of Cambridge, Edgar Douglas, Baron, 1941 (9), Trinity College, Cambridge, England

Alexandroff, Paul A., 1947 (1), Mathematical Institute of the Academy of Sciences of the U. S. S. R., Bolshaya Kalushskaya 19, Moscow, U. S. S. R.

Amaldi, Edoardo, 1962 (3), University of Rome, Piazzale delle Scienze, 5, Rome, Italy

Ambartsumian, Victor Amazaspovich, 1959 (2), Burakan Astronomical Observatory, Erevan, Armenia, U.S.S.R.

Bailey, Sir Edward, 1944 (6), 76 Hampstead Way, London, N. W. 11, England

Bartlett, Sir Frederic Charles, 1947 (12), 161 Huntingdon Road, Cambridge, England

Best, Charles Herbert, 1950 (9), Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

Bhabha, Homi Jehangir, 1963 (3), Tata Institute of Fundamental Research, Apollo Pier Road, Bombay 1, India

Born, Max, 1955 (3), Marcard Strasse 4, Bad Pyrmont, Germany

Bragg, Sir William Lawrence, 1945 (3), The Royal Institution, 21 Albemarle Street, London, W. 1, England

de Broglie, Prince Louis, 1948 (3), 94 Rue Perronet, Neuilly-sur-Seine, France

Brun, Edmond Antoine, 1960 (4), University of Paris, 8–10, place du Commerce, Paris XV, France

Bullard, Sir Edward Crisp, 1959 (13), Madingley Rise, Madingley Road, Cambridge, England

Bullen, Keith Edward, 1961 (13), Department of Applied Mathematics, University of Sydney, Sydney, Australia

Burnet, Sir Macfarlane, 1954 (10), The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Caso, Alfonso, 1943 (11), Avenida Central 234, Tlacopac, Villa Obregón, Mexico 20, D. F.

Chapman, Sydney, 1946 (13), High Altitude Observatory, Boulder, Colorado, U. S. A.

Clark, Sir Wilfrid Le Gros, 1963 (11), Department of Human Anatomy, Oxford University, South Parks Road, Oxford, England

Dale, Sir Henry Hallett, 1940 (9), The Wellcome Trust, 52 Queen Anne Street, London, W. 1, England

Dirac, Paul Adrien Maurice, 1949 (1), Department of Mathematics, St. John's College, Cambridge, England

Ephrussi, Boris, 1961 (7), Developmental Biology Center, Western Reserve University, 2127 Cornell Road, Cleveland 6, Ohio, U.S.A.

Eskola, Pentti Eelis, 1951 (6), Helsinki University, Snellmanink. 5, Helsinki, Finland

Florey, Sir Howard Walter, 1963 (10), The Royal Society, Burlington House, London, W. 1, England

von Frisch, Karl, 1951 (8), The Zoological Institute, University of Munich, Munich, Germany

Geijer, Per, 1958 (6), Agnevaegen 5, Djursholm I, Sweden

Hadamard, Jacques, 1926 (1), 12 Rue Emile Faguet, Paris XIV, France

Heisenberg, Werner, 1961 (3), Max Planck Institut für Physik und Astrophysik, Aumeisterstrasse 6, Munich 23, Germany

Hill, Archibald Vivian, 1941 (9), 16 Bishopswood Road, Highgate, London, N.6. England

Hinshelwood, Sir Cyril Norman, 1960 (5), Department of Chemistry, Exeter College, Oxford, England

Hodge, Sir William Vallance Douglas, 1959 (1), The Master's Lodge, Pembroke College, Cambridge, England

Hopf, Heinz, 1957 (1), Swiss Federal Institute of Technology, Zurich, Switzerland

Houssay, Bernardo Alberto, 1940 (9), Viamonte 2790, Buenos Aires, Argentina

Jeffreys, Sir Harold, 1945 (13), St. John's College, Cambridge, England

Kapitza, Peter Leonidovich, 1946 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Karrer, Paul, 1945 (5), Spyristeig 30, Zurich 7, Switzerland

Kihara, Hitoshi, 1958 (7), National Institute of Genetics, Misima, Japan

Konorski, Jerzy, 1963 (12), Department of Neurophysiology, Nencki Institute of Experimental Biology, 3, Pasteur Street, Warsaw 22, Poland

Kuno, Hisashi, 1963 (6), Geological Institute, University of Tokyo, Tokyo, Japan

Landau, Lev Davidovich, 1960 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Leloir, Luis F., 1960 (14), Instituto de Investigaciones Bioquimicas, Obligado 2490, Buenos Aires, Argentina

Levi, Giuseppe, 1940 (8), Instituto di Anatomia Umana, Corso Massimo D'Azeglio, 52, Turin, Italy

Lim, Robert K. S., 1942 (9), Medical Sciences Research Laboratory, Miles Laboratories, Inc., Elkhart, Indiana, U. S. A.

Lindblad, Bertil, 1955 (2), Stockholm Observatory, Saltsjöbaden, Sweden

Lwoff, André, 1955 (9), Institut Pasteur, Paris XV, France

Lynen, Feodor, 1962 (14), Max-Planck-Institut für Zellchemie, Karlstrasse 23–25, Munich 2, Germany

Michotte, Albert Edouard (Baron Michotte van den Berck), 1956 (12), University of Louvain, Louvain, Belgium

Mott, Sir Nevill Francis, 1957 (3), University of Cambridge, Cavendish Laboratory, Free School Lane, Cambridge, England

Oort, Jan Hendrik, 1953 (2), Observatory of Leiden, Leiden, The Netherlands

Penfield, Wilder, 1953 (10), Montreal Neurological Institute, 3801 University Street, Montreal 2, Canada

Penney, Sir William George, 1962 (4), United Kingdom Atomic Energy Authority, Charles II Street, London, S. W. 1, England

Piéron, Henri, 1949 (12), Institut de Psychologie, 28, rue Serpente, Paris VI, France

Prelog, Vladimir, 1961 (5), Laboratorium für organische Chemie, Eidgenossische Technische Hochschule, Zurich, Switzerland

Reichstein, Tadeus, 1952 (5), Organisch-chemische Anstalt, St. Johanns-Ring 19, Basel, Switzerland

Robertson, Rutherford **Ness,** 1962 (7), Department of Botany, University of Adelaide, Adelaide, South Australia

Robinson, Sir Robert, 1934 (5), 170 Piccadilly, London, W. 1, England

Ruzicka, Leopold, 1944 (5), Department of Organic Chemistry, Institute of Technology, Zurich, Switzerland

Semenov, Nikolai Nikolaevich, 1963 (5), Institute of Chemical Physics, Vorobyevskoye chaussee 2, Moscow, V-133, U.S.S.R.

Southwell, Sir Richard Vynne, 1943 (4), The Old House, Trumpington, Cambridge, England

Svedberg, The, 1945 (5), Fysikalisk-Kemiska Institutionen, Uppsala University, Uppsala, Sweden

Taylor, Sir Geoffrey Ingram, 1945 (1), Trinity College, Cambridge, England

Theorell, Axel **Hugo,** 1957 (14), Nobel Institute of Medicine, Solnavagen 1, Stockholm 60, Sweden

Tiselius, Arne W. K., 1949 (14), Institute of Biochemistry, Uppsala University, Uppsala, Sweden

Todd, of **Trumpington,** Alexander Robertus, Baron, 1955 (5), University of Cambridge, University Chemical Laboratory, Lensfield Road, Cambridge, England

Vallée-Poussin, C. de la, 1929 (1), 42, Avenue du Houx, Boitsfort, Belgium

Vening Meinesz, Felix **A**ndries, 1939 (13), Potgieterlaan 5, Amersfoort, The Netherlands

Watson, D. M. S., 1938 (8), University College, Gower Street, London, W.C. 1, England

Winge, Öjvind, 1949 (14), Department of Physiology, Carlsberg Laboratory, Copenhagen (Valby), Denmark

Yukawa, Hideki, 1949 (3), Yukawa Hall, Kyoto University, Kyoto, Japan

Number of Foreign Associates July 1, 1963: 68.

SECTIONS

(1) *Mathematics*—43 members

Whitney, Hassler, *Chairman* (1964)
Ahlfors, L. V.
Albert, A. A.
Alexander, J. W.
Bochner, S.
Brauer, R. D.
Chern, S. S.
Courant, R.
Doob, J. L.
Douglas, Jesse
Eilenberg, S.
Eisenhart, L. P.
Evans, G. C.
Feller, William
Friedrichs, K. O.
Gödel, Kurt
Hille, Einar
Jacobson, Nathan
Lefschetz, Solomon
Lin, C. C.
Mackey, George W.
Mac Lane, Saunders
McShane, E. J.
Milnor, J. W.
Montgomery, Deane
Moore, R. L.
Morrey, C. B., Jr.
Morse, Marston
Murnaghan, F. D.
Neyman, Jerzy
Shannon, C. E.
Smith, Paul A.
Spencer, D. C.
Steenrod, N. E.
Stoker, J. J.
Stone, M. H.
Thomas, T. Y.
Tukey, John W.
Walsh, J. L.
Whyburn, G. T.
Wilder, R. L.
Zariski, O.
Zygmund, Antoni

Foreign Associates

Alexandroff, P. A.
Dirac, P. A. M.
Hadamard, Jacques
Hodge, Sir William
Hopf, Heinz
Taylor, Sir Geoffrey
Vallée-Poussin, C. de la

(2) *Astronomy*—30 members

Menzel, D. H., *Chairman* (1965)
Abbot, C. G.
Aller, L. H.
Babcock, H. D.
Babcock, H. W.
Bowen, I. S.
Brouwer, Dirk
Chandrasekhar, S.
Clemence, G. M.
Goldberg, Leo
Greenstein, J. L.
Herget, Paul
Joy, A. H.
Kuiper, G. P.
Mayall, N. U.
Minkowski, R. L.
Morgan, W. W.
Nicholson, S. B.
Sandage, A. R.
Schwarzschild, M.
Seares, F. H.
Shane, C. D.
Shapley, Harlow
Slipher, V. M.
Spitzer, Lyman, Jr.
Stebbins, Joel
Tousey, Richard
Whipple, F. L.
Whitford, A. E.
Wilson, Olin C.

Foreign Associates

Ambartsumian, V.
Lindblad, Bertil
Oort, Jan Hendrik

(3) *Physics*—96 members

Anderson, C. D., *Chairman* (1966)
Allison, S. K.
Alvarez, L. W.
Anderson, H. L.
Bacher, R. F.
Bainbridge, K. T.
Bardeen, John
Beams, J. W.
Bethe, H. A.
Birge, R. T.
Bleakney, Walker
Bloch, Felix
Bloembergen, N.
Bradbury, N. E.
Brattain, W. H.
Breit, Gregory
Brillouin, Leon
Brode, R. B.
Chamberlain, Owen
Chew, Geoffrey F.
Condon, E. U.
Dennison, D. M.
Deutsch, M.
DuBridge, L. A.
DuMond, J. W. M.
Dunning, J. R.
Epstein, P. S.
Fairbank, W. M.
Feynman, R. P.
Fowler, W. A.
Franck, James
Gamow, George

Gell-Mann, Murray
Glaser, Donald A.
Goldberger, M. L.
Goldhaber, M.
Goudsmit, S. A.
Herb, R. G.
Herzfeld, K. F.
Hofstadter, R.
Houston, W. V.
Hull, A. W.
Inghram, Mark G.
Kemble, E. C.
Kerst, D. W.
Kittel, C.
Kusch, P.
Lamb, W. E., Jr.
Land, E. H.
Lauritsen, C. C.
Loomis, F. W.
Marshak, R. E.
Mayer, M. G.
McMillan, E. M.
Meggers, W. F.
Morse, P. M.
Mulliken, R. S.
Nier, A. O. C.
O'Brien, Brian
Oppenheimer, J. R.
Pais, A.
Panofsky, W. K. H.
Pound, R. V.
Purcell, E. M.
Rabi, I. I.
Ramsey, N. F.
Rossi, Bruno
Schiff, L. I.
Schwinger, Julian
Segrè, E.
Seitz, Frederick
Serber, R.
Simpson, J. A.
Slater, J. C.
Stern, Otto
Street, J. C.
Szilard, Leo
Teller, Edward
Thomas, L. H.
Townes, C. H.
Tuve, M. A.
Uhlenbeck, G. E.
Van Vleck, J. H.
Webster, D. L.
Weinberg, Alvin M.
Weisskopf, V. F.
Wentzel, Gregor
Wheeler, J. A.
Wick, Gian-Carlo
Wigner, E. P.
Williams, John H.
Wilson, Edwin B.
Wilson, Robert R.
Wu, C. S.
Zacharias, J. R.
Zachariasen, W. H.

Foreign Associates

Amaldi, Edoardo
Bhabha, Homi J.
Born, Max
Bragg, Sir Lawrence
de Broglie, Prince Louis
Heisenberg, Werner
Kapitza, P. L.
Landau, L. D.
Mott, Sir Nevill
Yukawa, Hideki

(4) *Engineering*—50 members

Sherwood, T. K., *Chairman* (1965)
Astin, A. V.
Bain, E. C.
Benedict, Manson
Bode, H. W.
Brooks, Harvey
Bush, Vannevar
Chipman, John
Curme, G. O., Jr.
Darken, L. S.
Den Hartog, J. P.
Draper, C. S.
Dryden, H. L.
Fisk, J. B.
Fletcher, Harvey
Foote, P. D.
Gibbs, W. F.
Gilliland, E. R.
Greenewalt, C. H.
Hoover, Herbert
Hottel, H. C.
Hunsaker, J. C.
Jeffries, Zay
Kelly, M. J.
Kinzel, A. B.
Lewis, W. K.
Loomis, A. L.
Mehl, R. F.
Olson, Harry F.
Pickering, W. H.
Pierce, J. R.
Piore, E. R.
Raymond, A. E.
Savage, J. L.
Shockley, W.
Slepian, Joseph
Smith, C. S.
Soderberg, C. R.
Sporn, Philip
Stratton, J. A.
Suits, C. G.
Terman, F. E.
Thomas, C. A.
Timoshenko, Stephen
Wiesner, J. B.
Wilson, Robert E.
Wolman, Abel
Zener, Clarence
Zinn, W. H.
Zworykin, V. K.

Foreign Associates

Brun, Edmond A.
Penney, Sir William
Southwell, Sir Richard

(5) *Chemistry*—98 members

Niemann, Carl, *Chairman* (1965)
Adams, Roger
Badger, R. M.
Baker, W. O.
Bartlett, P. D.
Blomquist, A. T.
Boekelheide, V.
Bolton, E. K.
Brewer, Leo
Brode, W. R.
Brown, H. C.
Calvin, Melvin
Conant, J. B.
Connick, R. E.
Cope, A. C.
Cram, Donald J.
Crawford, Bryce, Jr.
Daniels, Farrington
Davidson, N.
Debye, Peter
Djerassi, Carl
Doering, William
Elderfield, R. C.
Emmett, P. H.
Eyring, Henry
Ferry, John D.
Fieser, L. F.
Flory, P. J.
Folkers, Karl
Fuoss, R. M.
Fuson, R. C.
Gates, M.
Giauque, W. F.
Gilman, Henry
Gutowsky, H. S.
Hammett, L. P.
Hammond, G. S.
Harned, H. S.
Hauser, C. R.
Hendricks, S. B.
Hildebrand, J. H.
Hirschfelder, J. O.
Hornig, D. F.
Hutchison, C. A., Jr.
Jacobs, W. A.
Johnson, J. R.
Johnson, W. S.
Keyes, F. G.
Kimball, G. E.
Kistiakowsky, G. B.
Kolthoff, I. M.
Kraus, C. A.
La Mer, V. K.
Leonard, N. J.
Libby, W. F.
Lind, S. C.
Lipscomb, W. N.
Long, Franklin A.
Longsworth, L. G.
MacInnes, D. A.
Mark, H. F.
Marvel, C. S.
Mayer, J. E.
McElvain, S. M.
Newman, M. S.
Noyes, W. A., Jr.
Onsager, Lars
Pauling, Linus
Perlman, Isadore
Pitzer, K. S.
Roberts, John D.
Rossini, F. D.
Scatchard, George
Seaborg, G. T.
Shedlovsky, Theodore
Sheehan, J. C.
Smith, L. I.
Smyth, C. P.
Spedding, F. H.
Stockmayer, W. H.
Stork, Gilbert
Tarbell, D. S.
Taube, Henry
Tishler, Max
Urey, H. C.
Wall, Frederick T.
Warner, J. C.
Westheimer, F. H.
Williams, J. W.
Williams, Robert R.
Wilson, E. Bright, Jr.
Winstein, Saul
Wolfrom, M. L.
Woodward, R. B.
Wyckoff, R. W. G.
Yost, D. M.
Young, W. G.
Zimm, B. H.

Foreign Associates

Hinshelwood, Sir Cyril
Karrer, Paul
Prelog, Vladimir
Reichstein, Tadeus
Robinson, Sir Robert
Ruzicka, Leopold
Semenov, Nikolai N.
Svedberg, The
Lord Todd

(6) *Geology*—37 members

Anderson, C. A., *Chairman* (1966)
Abelson, P. H.
Allen, E. T.
Birch, Francis
Blackwelder, Eliot
Bradley, W. H.
Bramlette, M. N.
Bucher, W. H.
Buddington, A. F.
Buerger M. J.
Chaney, R. W.
Cloos, Ernst
Cloud, Preston E., Jr.
Dunbar, C. O.
Garrels, R. M.
Gilluly, James
Gregory, W. K.
Hedberg, H. D.
Hess, H. H.
Hewett, D. F.
Hubbert, M. King
James, Harold L.
Knopf, Adolph

Krauskopf, K. B.
Longwell, C. R.
Lovering, T. S.
Mackin, J. H.
Nolan, T. B.
Patterson, Bryan
Rubey, W. W.
Russell, R. J.
Schairer, J. F.
Simpson, G. G.
Turner, F. J.
Williams, Howel
Woodring, W. P.
Yoder, H. S., Jr.

Foreign Associates

Bailey, Sir Edward
Eskola, Pentti
Geijer, Per
Kuno, Hisashi

(7) *Botany*—49 members

Thimann, K. V., *Chairman* (1965)
Anderson, Edgar
Arnold, William A.
Arnon, Daniel I.
Barker, H. A.
Beadle, G. W.
Benzer, Seymour
Blinks, L. R.
Bonner, David M.
Bonner, James
Borthwick, H. A.
Braun, A. C.
Brink, R. A.
Burkholder, P. R.
Burris, R. H.
Chandler, W. H.
Clausen, Jens
Cleland, R. E.
Couch, J. N.
Delbrück, Max
Esau, Katherine
Fred, E. B.
French, C. S.
Goddard, D. R.
Hershey, A. D.
Hollaender, A.
Horsfall, J. G.
Kaufmann, B. P.
Kramer, Paul J.
Luria, S. E.
Mangelsdorf, P. C.
McClintock, Barbara
Osterhout, W. J. V.
Raper, K. B.
Rhoades, M. M.
Riker, A. J.
Robbins, W. J.
Roberts, R. B.
Sax, Karl
Sinnott, E. W.
Skoog, Folke
Smith, A. C.
Stakman, E. C.
Stebbins, G. L.
Van Niel, C. B.
Walker, J. C.
Went, F. W.
Wetmore, R. H.
Wilson, P. W.

Foreign Associates

Ephrussi, Boris
Kihara, H.
Robertson, R. N.

(8) *Zoology and Anatomy*—63 members

Mayr, Ernst, *Chairman* (1964)
Bartelmez, G. W.
Bigelow, H. B.
Bloom, William
Bodenstein, D. H.
Bodian, David
Briggs, Robert
Bullock, T. H.
Burns, R. K.
Cleveland, L. R.
Colbert, E. H.
Corner, G. W.
Crow, James F.
Danforth, C. H.
Demerec, Milislav
Dobzhansky, Th.
Dunn, L. C.
Emerson, Alfred E.
Friedmann, Herbert
Glass, Bentley
Griffin, D. R.
Hamburger, Viktor
Haskins, C. P.
Hisaw, F. L.
Holtfreter, J.
Hubbs, C. L.
Hutchinson, G. E.
Hyman, Libbie
Irwin, M. R.
Jacobs, M. H.
Kellogg, Remington
Lerner, I. M.
Lewis, W. H.
Mazia, Daniel
Metz, C. W.
Miller, A. H.
Mirsky, A. E.
Moore, J. A.
Muller, H. J.
Neel, J. V.
Nicholas, J. S.
Painter, T. S.
Palade, G. E.
Petrunkevitch, A.
Pittendrigh, C. S.
Riddle, Oscar
Romer, A. S.
Schmidt-Nielsen, K.
Schmitt, F. O.
Scholander, P. F.
Smith, Philip E.
Sonneborn, T. M.
Stern, Curt

Stone, W. S.
Straus, W. L., Jr.
Sturtevant, A. H.
Twitty, V. C.
Weiss, Paul
Wetmore, Alexander
Williams, C. M.
Willier, B. H.
Wright, Sewall
Zirkle, R. E.

Foreign Associates

von Frisch, Karl
Levi, Giuseppe
Watson, D. M. S.

(9) *Physiology*—31 members

Davis, Hallowell, *Chairman* (1966)
Astwood, E. B.
Aub, Joseph C.
Bard, Philip
Brink, Frank, Jr.
Bronk, Detlev W.
Cole, K. S.
Comroe, Julius H., Jr.
Cournand, André
Erlanger, Joseph
Evans, H. M.
Fenn, W. O.
Forbes, Alexander
Gerard, R. W.
Hartline, H. K.
Hastings, A. B.
Ingle, D. J.
Kety, Seymour S.
Landis, E. M.
Lloyd, D. P. C.
Loeb, R. F.
Long, C. N. H.
Lorente de Nó, R.
Magoun, H. W.
Marshall, E. K., Jr.
Pitts, R. F.
Richards, A. N.
Richards, D. W.
Schmidt, C. F.
Visscher, M. B.
Woolsey, C. N.

Foreign Associates

Lord Adrian
Best, C. H.
Dale, Sir Henry
Hill, A. V.
Houssay, B. A.
Lim, Robert K. S.
Lwoff, André

(10) *Pathology and Microbiology*—46 members

Francis, Thomas, Jr., *Chairman* (1966)
Armstrong, Charles
Bailey, P.
Blalock, Alfred
Cannon, P. R.
Castle, W. B.
Coggeshall, L. T.
Coons, Albert H.
Dalldorf, Gilbert
Dingle, J. H.
Dochez, A. R.
Dragstedt, L. R.
Dubos, R. J.
Dulbecco, Renato
Eagle, Harry
Enders, J. F.
Goebel, W. F.
Heidelberger, Michael
Horsfall, F. L., Jr.
Hotchkiss, R. D.
Huebner, R. J.
Huggins, C. B.
Little, C. C.
Long, E. R.
MacLeod, C. M.
Maxcy, K. F.
McCarty, Maclyn
McMaster, P. D.
Meyer, K. F.
Miller, C. P.
Opie, E. L.
Paul, John R.
Puck, T. T.
Rich, A. R.
Robertson, O. H.
Rous, Peyton
Sabin, Albert B.
Shope, R. E.
Smadel, J. E.
Taliaferro, W. H.
Tillett, W. S.
Waksman, S. A.
Warren, Shields
Watson, C. J.
Whipple, G. H.
Wood, W. Barry, Jr.

Foreign Associates

Burnet, Sir Macfarlane
Florey, Sir Howard
Penfield, Wilder

(11) *Anthropology*—13 members

Hallowell, A. Irving, *Chairman* (1966)
Albright, W. F.
Coon, C. S.
Eggan, Fred
Haury, E. W.
Lothrop, S. K.
Movius, H. L., Jr.
Rouse, Irving
Shapiro, H. L.
Steward, J. H.
Stewart, T. D.
Washburn, S. L.
Willey, G. R.

Foreign Associates

Caso, Alfonso
Clark, Sir Wilfrid Le Gros

(12) *Psychology*—24 members

Hilgard, E. R., *Chairman* (1965)
Beach, F. A.
Boring, E. G.
Carmichael, Leonard
Estes, W. K.
Galambos, R.
Graham, C. H.
Guilford, J. P.
Harlow, H. F.
Klüver, Heinrich
Köhler, Wolfgang
Lindsley, D. B.
Miles, W. R.
Miller, George A.
Miller, N. E.
Pfaffmann, Carl
Richter, C. P.
Riggs, Lorrin A.
Skinner, B. F.
Spence, K. W.
Sperry, R. W.
Stevens, S. S.
v. Békésy, Georg
Wever, E. G.

Foreign Associates

Bartlett, Sir Frederic
Konorski, Jerzy
Michotte, Albert E.
Piéron, Henri

(13) *Geophysics*—35 members

Revelle, Roger, *Chairman* (1966)
Adams, L. H.
Benioff, Hugo
Berkner, L. V.
Bjerknes, J.
Booker, H. G.
Brown, Harrison
Byerly, Perry
Byers, H. R.
Eckart, Carl
Elsasser, W. M.
Ewing, Maurice
Forbush, Scott E.
Friedman, H.
Griggs, D. T.
Gunn, Ross
Haurwitz, B.
Iselin, C. O'D.
Kaplan, Joseph
Knopoff, Leon
Lambert, W. D.
MacDonald, G. J. F.
Munk, W. H.
Pekeris, C. L.
Piggot, C. S.
Press, Frank
Redfield, A. C.
Reichelderfer, F. W.
Slichter, L. B.
Stommel, Henry
Van Allen, J. A.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
Wulf, O. R.

Foreign Associates

Bullard, Sir Edward
Bullen, Keith Edward
Chapman, Sydney
Jeffreys, Sir Harold
Vening Meinesz, F. A.

(14) *Biochemistry*—58 members

Carter, H. E., *Chairman* (1966)
Anfinsen, C. B.
Ball, E. G.
Balls, A. K.
Bloch, K. E.
Buchanan, John M.
Chance, Britton
Clark, W. M.
Clarke, H. T.
Cori, Carl F.
Craig, L. C.
Doisy, E. A.
Doty, Paul
Doudoroff, Michael
du Vigneaud, V.
Edsall, J. T.
Fruton, J. S.
Green, David E.
Hassid, W. Z.
Hofmann, Klaus
Horecker, B. L.
Kalckar, H. M.
Kamen, Martin D.
Kendall, E. C.
King, C. G.
Kornberg, A.
Lardy, Henry
Lederberg, Joshua
Lehninger, A. L.
Link, K. P.
Lipmann, Fritz
Maynard, L. A.
McCollum, E. V.
McElroy, W. D.
Moore, Stanford
Neurath, Hans
Northrop, J. H.
Ochoa, Severo
Oncley, J. L.
Rittenberg, David
Rose, W. C.
Shemin, David
Smith, Emil L.
Snell, E. E.
Stanley, W. M.
Stein, W. H.
Szent-Györgyi, A.
Tatum, E. L.
Van Slyke, D. D.
Vickery, H. B.
Wald, George
Watson, J. D.
Williams, R. C.
Williams, R. J.
Wilson, D. W.
Wintersteiner, O.
Wood, H. G.
Woolley, D. W.

Foreign Associates

Leloir, Luis F.
Lynen, Feodor
Theorell, Hugo
Tiselius, Arne W. K.
Winge, Öjvind

INFORMATION TO CONTRIBUTORS

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